Early neurogenesis in the flour beetle *Tribolium castaneum*

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Abstract

Insects exhibit considerable variety in their morphology and can be found in many diverse habitats. Despite these variations, early neurogenesis seems to be conserved in insects. In all species investigated to date it begins with the formation of neural stem cells (neuroblasts), which establish a distinct internal layer and produce a fixed number of neurons and glial cells. The neuronal cells then form a characteristic rope ladder-like axonal scaffold. However, it is evident that the composition or identity of the individual neurons must have changed during insect evolution to allow for variations in neuronal networks. This raises questions regarding which developmental steps have been changed and the manner in which they have been modified. In order to address these questions, early neurogenesis was analysed in the flour beetle *Tribolium castaneum* and the results were compared to the well-studied fruit fly *Drosophila melanogaster*. Initially a map of trunk neuroblasts in *T. castaneum* was established, which revealed a high degree of conservation in the arrangement of individual neuroblasts compared to *D. melanogaster*. However, a comparison of the expression patterns of genes that confer regional identity to neuroblasts showed considerable variations. Significant differences in the expression patterns of the segment polarity gene *wingless* and the columnar gene *ventral nerve cord defective* (*vnd*) were found. Furthermore, the impact these changes in neuroblast identity have on the composition and identity of their respective progeny was analysed. As a result changes in the number of Even-skipped and Tailup expressing neurons in *T. castaneum* embryos were found, with three-fold more Tailup expressing neurons compared to *D. melanogaster*. To further analyse the role of the neuroblast identity gene *vnd* in the formation of Even-skipped positive neurons, RNAi gene silencing studies were performed, resulting in the loss of neurons and changes in neuronal migration pattern. In summary, the results demonstrate that evolutionary changes in neuronal networks result from changes in neuroblast identity, which in turn have an impact on the composition of neuronal lineages.

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Abbreviations

A1-A10	abdominal segments 1-10
aCC	anterior corner cell
AEL	after egg laying
AP	anterio-posterior
At	antenna
CNS	central nervous system
DV	dorsal-ventral
El cluster	Eve lateral cluster
For	forward
GMC	ganglion mother cell
GS	rudiment for gnathal segments
GZ	growth zone
HG	hindgut
HL	head lobes
ISN	intersegmental nerve
La	labium
Lb	labrum
Mn	mandible
Mx	Maxilla
NB	Neuroblast
NPG	neural precursor group
NS	nervous system stage
pCC	posterior corner cell
Pd	Proctodeum
PEL	posterior Eve lateral cluster
Pl	Pleuropodia
Rev	Reverse
RT	room temperature
SN	segmental nerve
St	Stomodeum
T1	thoracic segment 1
T2	thoracic segment 2
T3	thoracic segment 3
TS	rudiment for thoracic segments
VNC	ventral nerve cord

1 Introduction

Arthropods are the most abundant animal group on our planet and occupy marine, freshwater, terrestrial and aerial environments. They comprise the insects, crustaceans, chelicerates and myriapods. Despite the enormous variety in morphology observed within the group, there are a number of characteristics which are common to all arthropods. One of those shared features is the organisation of the nervous system into segmental ganglia, resulting in its characteristic rope-ladder like appearance. Nevertheless, despite the conserved appearance, the nervous system must be adapted to the individual species specific morphology and behaviour. Therefore modifications of developmental processes involved in generating the nervous system are expected to have evolved over time between different groups and species. In recent years data on the early development and structure of the nervous system in all major arthropod groups have been generated (review see Stollewerk and Simpson 2005 and Stollewerk 2008, Bate 1976, Hartenstein and Campos-Ortega 1984, Doe and Goodman 1985, Truman and Ball 1998, Wheeler *et al.* 2003: insects, Stollewerk *et al.* 2001, Doeffinger and Stollewerk 2010: chelicerates, Dove and Stollewerk 2003, Kadner and Stollewerk 2004, Chipman and Stollewerk 2006: myriapods, Dohle 1976, Scholtz 1992, Gerberding 1997, Harzsch 2001, Ungerer and Scholtz 2008, Ungerer *et al.* 2011: crustaceans). However, molecular data on related species in the individual arthropod groups is rare. The best studied arthropod in terms of nervous system development is, without doubt, the fruit fly *Drosophila melanogaster* (Diptera; Drosophilidae). Aspects of the development of the nervous system have been studied in a number of other insect species, but not to the same degree of detail as in *Drosophila*.

The flour beetle *Tribolium castaneum* (Coleoptera, Tenebrionidae) provides a valuable organism with which to compare early neurogenesis within insects. *Tribolium's* strengths – easily bred, long lifespan, short life cycle, high fecundity, stocks require little care (Sokoloff 1972), have recently led it become a model organism in evolutionary developmental biology (Brown *et al.* 2009). In contrast to *Drosophila melanogaster* it exhibits developmental processes of a more basal character for insects (Brown *et al.* 2009). For example, *Tribolium* develops a proper larval head (Bucher and Wimmer 2005)

and possesses external larval appendages. Furthermore, its segmentation develops sequentially from anterior to posterior, known as short-germ development. This development is common for hemimetabolous insects (*e. g.* grasshopper) but not for all holometabolous insects (Chapman 1998). For example the holometabolous fruit fly *Drosophila* undergoes long-germ-band development, during which all segments form simultaneously. These features, as well as the recently sequenced genome of *Tribolium* (Richards *et al.* 2008) and the growing number of molecular tools available make it a valuable model organism with which to gain better insight into the modification of neural development in insects.

In the current work specific aspects of early neurogenesis in the flour beetle *Tribolium castaneum* were investigated, thus providing a basis to compare mechanisms of early neurogenesis with the fruit fly *Drosophila melanogaster*. Where possible the comparison was extended to the basally branching insect *Ctenolepisma longicaudata* (Zygentoma; Lepismatidae) and the hemimetabolous insect *Schistocerca* (Orthoptera; Acrididae).

To allow an understanding of the phylogenetic relationships between the discussed species a short and simplified overview on the relative position of each species within the arthropods is provided in the following paragraph (see Fig. 1-1) (for review see Trautwein *et al.* 2012). Traditionally, based on morphological evidence, myriapods and hexapods were grouped together in the group of Tracheata (Kraus 2001). In recent years, molecular data and the “reinterpretation of morphological evidence” (Trautwein *et al*. 2012) support the idea that crustaceans and hexapods form the monophyletic group of Pancrustacea (Giribet *et al.* 2001, Regier *et al.* 2005, Meusemann *et al.* 2010, Rota-Stabelli *et al.* 2011). Which of the crustacean lineages forms the sister group of the Hexapoda is, however, still controversial (Trautwein *et al.* 2012). Hexapods comprise the insects and the entognathes (hexapods with enclosed jaws, *e. g.* Collembola) (Klass and Kristensen 2001). The relationships within the Entognatha and their exact relationship in respect to the insects are not resolved, yet (Carapelli *et al.* 2007, Trautwein *et al.* 2012). Within the insects, the wingless Zygentoma (silverfish) are one of the two earliest lineages branching of (Trautwein *et al.* 2012). The remaining insects comprise the monophyletic group of Neoptera, which consists of the Polyneoptera (for example the Orthoptera), the Paraneoptera and the Holometabola. The monophyly of holometabolous insects is well

supported with the Hymenoptera (wasps, bees, ants) being the earliest diverging lineage, forming the sister group to all other holometabolous insects. The remaining holometabolous insects are grouped into the Neuropteroidea (for example Coleoptera) and the Mecopterida (for example Diptera) (for review see Trautwein *et al.* 2012).

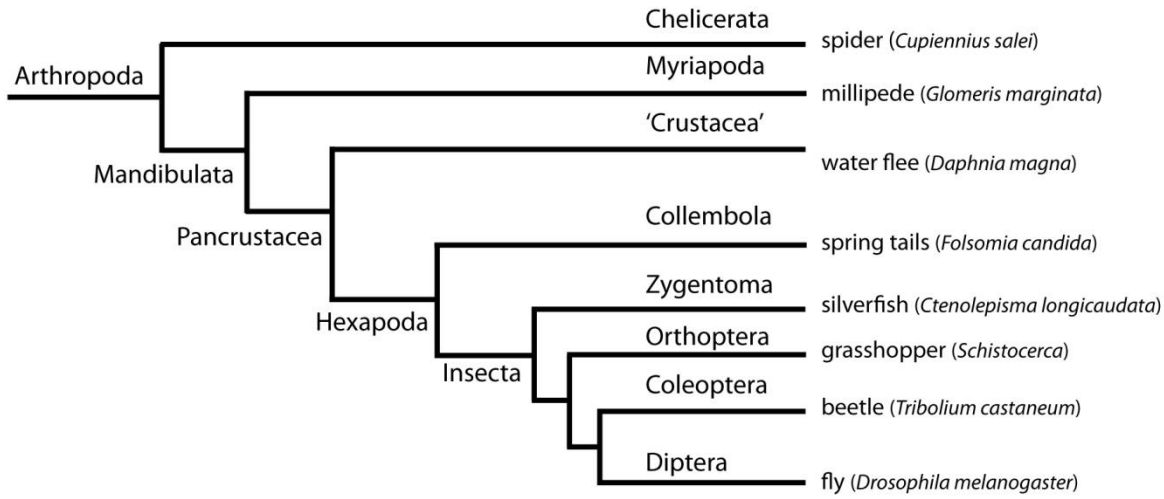

Figure 1-1: Simplified phylogeny of arthropods

The figure provides a simplified phylogeny of relevant arthropod groups, and is intended to provide an overview on the relative position within arthropods of the species discussed. Relationships of groups are based on Regier *et al.* (2010) and Trautwein *et al.* (2012).

In the following section the current literature concerning mechanisms during early neurogenesis of all four groups of arthropods, with special emphasis on *Drosophila*, will be reviewed, demonstrating similarities and differences which have evolved.

Formation of the nervous system in arthropods with special emphasis on *Drosophila melanogaster*

The central nervous system (CNS) of the *Drosophila* embryo consists of the brain and the ventral nerve cord (VNC). Fourteen similar segmental units, called neuromeres, form the VNC (three gnathal, three thoracic and eight abdominal). A neuromere is further divided into two hemineuromeres, which are separated by midline cells (Fig. 1-2 A). The VNC develops out of the neurogenic region (also referred to as neuroectoderm) in the embryo which is defined as the “medial ectodermal region, from which neuroblasts segregate” (Hartenstein and Campos-Ortega 1984). The neurogenic region is positioned at the

ventral side of the embryo and consists of ectodermal cells which either adopt the fate of neural precursor cells (neuroblasts) or that of epidermal precursor cells (Campos-Ortega and Hartenstein 1985, Lehman *et al.* 1981). Neural cell-fate is determined by the proneural genes *achaete, scute* and *lethal of scute* which are combined in the Achaete-Scute Complex (AS-C) (Cabrera *et al.* 1987, Romani *et al.* 1989, Jimenez and Campos-Ortega 1990). A further gene which is part of the AS-C is the neural precursor gene *asense* which is only expressed in the neural precursors itself (Brand *et al.* 1993). The proneural genes of the AS-C are expressed in clusters of ectodermal cells in the neuroectoderm, called proneural clusters, at the beginning of neurogenesis (Fig. 1-2 B, C). Only a single cell out of a proneural cluster differentiates into a neural neuroblast with all the remaining cells adopting the fate of epidermoblasts (progenitor cells of epidermis). The neurogenic genes *Delta* and *Notch* narrow the expression of the proneural genes to a single cell in each proneural cluster through the mechanism of lateral inhibition (Lehman *et al.* 1983, Skeath and Carroll 1992, Heitzler *et al.* 1996) The thereby chosen neuroblast delaminates into a layer between ectoderm and mesoderm (Hartenstein and Campos-Ortega 1984). After reaching its final size the neuroblast divides asymmetrically in a stem cell manner (Stent and Weisblat 1985) budding off a ganglion mother cell (GMC) and renewing itself. The ganglion mother cell further divides into two neural cell types, either neurons or glial cells (Campos-Ortega and Hartenstein 1985, Goodman and Doe 1993).

In *Drosophila* 30 neuroblasts and the unpaired median neuroblast (MNB) delaminate out of the neurogenic region in each hemisegment within five segregation waves (S1 – S5), forming a characteristic pattern, between embryonic stage 8 to 11 (embryonic stages according to Campos-Ortega and Hartenstein 1985, see *Drosophila* staging Hartenstein 1993 in appendix). Neuroblasts are arranged in seven rows with around three to six cells each (Fig. 1-3 and 1-4; Hartenstein and Campos-Ortega 1984, Doe 1992). These neuroblasts are assigned a two number code according to their position in the hemisegment. The first number defines the row (one to seven) along the anterio-posterior (AP) axis. The second number defines the position of the neuroblast along the dorsal-ventral (DV) axis, with number one closest to the midline and number six most lateral (Fig. 1-4). *Drosophila* neuroblasts were originally named after the cells sharing equivalent positions in *Schistocerca* (Doe 1992), frequently referred to in the literature as positional homologues.

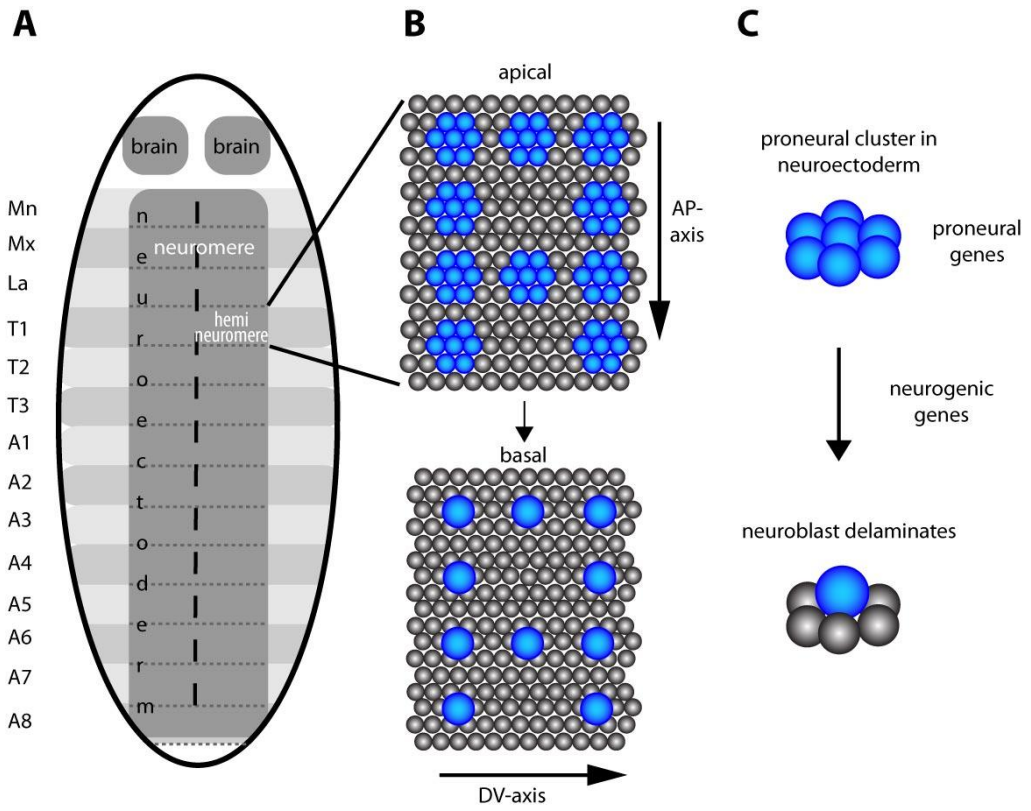

Figure 1-2: Early neurogenesis in *Drosophila*

(A) *Drosophila* embryo depicting the division into 14 segments. The VNC develops out of the neuroectoderm which is positioned ventrally. The part of the segment which forms the nervous system is called neuromere and is further divided by the midline (vertical dashed line) into two bilateral hemineuromeres. (B) One hemineuromere during the initial phase of neuroblast formation. Ten proneural clusters are initially formed in the neuroectoderm (blue cells) out of which the first neuroblasts arise during S1. (C) Initially all cells of a proneural cluster express proneural genes enabling them to differentiate into neuroblasts. The neurogenic genes narrow the expression to a single cell, which then delaminates inside the embryo.

Proneural gene expression and their function in the formation of neuroblasts is conserved between *Drosophila* and *Tribolium* (Wheeler *et al.* 2003). *Tribolium* possesses an Achaete- scute complex consisting of the single proneural gene *Tc-achaete-scute homolog* (*Tc-ASH*) and the neural precursor gene *asense* (*Tc-ase*). *Tc-ASH* in *Tribolium* combines the function of the three genes of the ac/sc complex (*achaete*, *scute*, *l'scute*) in *Drosophila* and is expressed in proneural clusters and transiently in all neuroblasts. As in *Drosophila* the expression becomes restricted to one cell of the proneural cluster and RNAi studies have revealed that it is required for neuroblast formation (Wheeler *et al.*

2003). *Tc-ase* in contrast is exclusively expressed in neuroblasts similar to *Drosophila*. The expression of *Tc-ase* is initiated in neural precursor cells after their delamination from the proneural clusters and is maintained throughout neurogenesis, making it appropriate as a marker for identifying neuroblasts (Wheeler *et al.* 2003).

Only recently Kux *et al.* (2013) suggested that the mechanism of lateral inhibition is conserved between *Tribolium* and *Drosophila*. They analysed the expression of the Enhancer of split orthologues (*E(spl)1* and *E(spl)3*) which in *Drosophila* are activated by Notch and subsequently repress the proneural genes in the proneural clusters therein promoting epidermoblast development. Only the ectodermal cell in the proneural cluster with the least Notch signal and no E(spl) expression, develops into a neuroblast (Culi and Modolell 1998). As in *Drosophila* the *E(spl)* orthologues in *Tribolium* depend on *Notch* expression and are expressed in all cells of a proneural cluster except for the future neuroblast. Furthermore, silencing of *Notch* and *E(spl)* transcripts result in the formation of many more neuroblasts as in wild type resembling the “neurogenic” phenotype, first described in *Drosophila* (Lehman *et al.* 1983).

The nervous system of crustaceans develops from neuroblasts similar to *Drosophila*. However, these neuroblasts and the molecular mechanisms leading to their formation exhibit major differences. In crustaceans neuroblasts remain in the outer neuroepithelium and do not delaminate inside the embryo as in insects (Dohle 1976, Gerberding 1997, Scholtz 1992, Harzsch 2001, Ungerer and Scholtz 2008, Ungerer *et al.* 2011). Additionally malacostracan neuroblasts are competent to generate GMCs as well as epidermal precursor cells, switching from the formation of one type to the other (Dohle 1976, Scholtz 1990). Studies on the expression of proneural genes in *Daphnia magna* (Branchiopoda, Daphnidiae) demonstrate further differences. In contrast to *Drosophila*, where the proneural *ac/sc* genes are the first neural genes to be expressed in proneural clusters, in *Daphnia snail* is the first neural gene expressed in the nervous system followed by the expression of the single *ASH* homologue. Furthermore, no proneural clusters were detected in *Daphnia* (Ungerer and Scholtz 2008, Ungerer *et al.* 2011). However, the final arrangement of neuroblasts in columns and rows in crustaceans is similar to *Drosophila* (Fig. 1-3; Scholtz 1992, Ungerer and Scholtz 2008, Ungerer *et al.* 2012).

A noticeable difference between insect and crustacean neurogenesis and that in chelicerates and myriapods is the lack of neuroblast in the latter groups. Instead of a single neural precursor cell (the neuroblast) groups of neural precursor cells (NPGs) segregate inside the embryo and differentiate into neural progeny without further divisions (Stollewerk *et al.* 2001, Mittmann 2002, Dove and Stollewerk 2003). As in insects homologues of the proneural genes are required to initiate nervous system formation (Stollewerk *et al.* 2001, Dove and Stollewerk 2003, Kadner and Stollewerk 2004). The arrangement of the groups of neural precursor cells in chelicerates and myriapods is, however, strikingly similar to that of neuroblasts in insects, as they are arranged in seven rows and several columns (see Fig. 1-3; Stollewerk *et al.* 2001, Dove and Stollewerk 2003, Doeffinger and Stollewerk 2010).

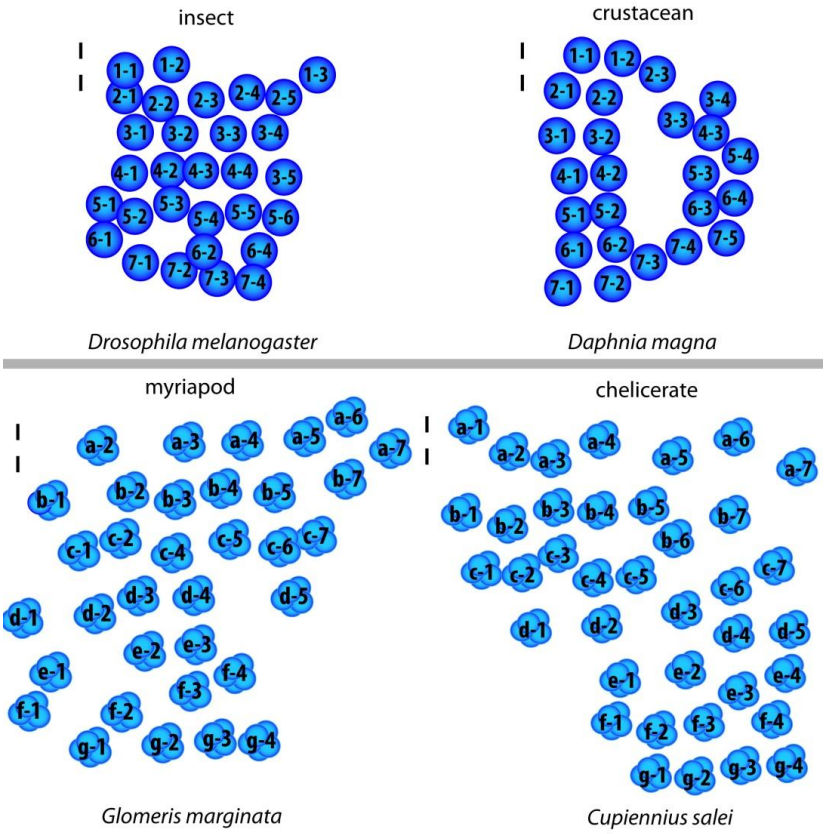

Figure 1-3: Arrangement of neural precursor cells in arthropods

Illustration of the arrangement of neural precursor cells in one hemineuromere in insects, crustaceans, myriapods and chelicerates. In insects and crustaceans single neural precursor cells (neuroblasts) are arranged in seven rows and three to six columns. In myriapods and chelicerates groups of neural precursor cells are arranged in a similar pattern. Rows are indicated by the letters a to g in order to prevent the assumption of homology between neuroblasts and groups of neural precursor cells. The dashed line indicates the midline. Figure modified after Doeffinger and Stollewerk (2010) and Ungerer *et al.* (2012).

History of insect neuroblasts

Neural precursor cells were first described in insects more than 100 years ago (Wheeler 1891). Wheeler called these neural precursor cells neuroblasts in his description of the nervous system development in the grasshopper *Xiphidium ensiferum*. He adopted the term from a description of neural precursor cells in annelids (Wheeler 1891). However, the first complete neuroblast map of an insect embryo (*Locusta migratoria*) was only published 85 years later (Bate 1976). At that time interest was focused primarily on the nervous system of adult insects and larvae (Bate 1976) and neuron maps for several insects, including the grasshopper *Schistocerca gregaria* and the cockroach *Periplaneta americana*, had been established which all showed a similar arrangement (Cohen and Jacklet 1967; Young 1969; Bentley 1970). Bate was interested in the mechanisms of how different neurons acquire their unique fate. To answer this question he established a map of neuroblasts in the thoracic and abdominal segments of *Locusta* in order to analyse the relationship with their neural progeny. Detailed neuroblast maps for further insects were published over the next 20 years (Hartenstein and Campos-Ortega 1984, Doe 1992: *Drosophila melanogaster*; Tamarelle *et al.* 1985: *Carausius morosus*, Doe and Goodman 1985a: *Schistocerca americana*, Shepherd and Bate 1990: *Schistocerca gregaria*, Breidbach and Urbach 1996: *Tenebrio molitor* (suboesophagal ganglia), Truman and Ball 1998: *Ctenolepisma longicaudata*). Additionally, Thomas *et al.* (1984) claim to have produced ‘detailed’ neuroblast maps for *Calliphora*, *Manduca* and *Rhodnius* reflecting the general pattern of seven rows of neural precursor cells in insects. The authors do not, however, provide further details on the exact arrangement and numbers of neuroblasts.

The first neuroblast maps to be produced were purely morphological descriptions (Bate 1976, Hartenstein and Campos-Ortega 1984, Doe and Goodman 1985a). Neuroblasts in the grasshopper are easily distinguished from other cells by their pale nuclei and their relatively large size and position (Wheeler, 1891). Cell size in grasshoppers originally favoured studies on grasshoppers over the fruit fly, with neuroblasts almost three times larger than in *Drosophila* and subsequently larger progenitor cells (neuroblast size in grasshopper = 20-30µm; Doe and Goodman 1985a). Advances in molecular techniques and their relative ease of application in *Drosophila* eventually led the fruit fly to emerge as the preferred model organism for analysis of insect neurogenesis (Thomas *et al.* 1984).

Early studies on phenotypes and development of mutants for early neurogenesis identified genes which are involved in neuroblast formation and differentiation (Lehman *et al.* 1981; Lehmann *et al.* 1983). Eventually gene expression patterns for all individual neuroblasts in a hemisegment were produced, thus allowing the unique identification of every neuroblast and many of its progenitor cells (Doe 1992; Broadus *et al.* 1995).

In *Tribolium* neuroblast formation and arrangement has not previously been described in detail. However, Wheeler *et al.* (2003) briefly mention the occurrence of sequential waves of neural precursor formation similar to that in *Drosophila*, which eventually results in seven anterio-posterior rows and three dorsal-ventral columns of neural precursors. In the present work the formation and arrangement of neuroblasts was analysed in greater detail, resulting in the production of a neuroblast map for *Tribolium* similar to those established for *Drosophila* and *Schistocerca*.

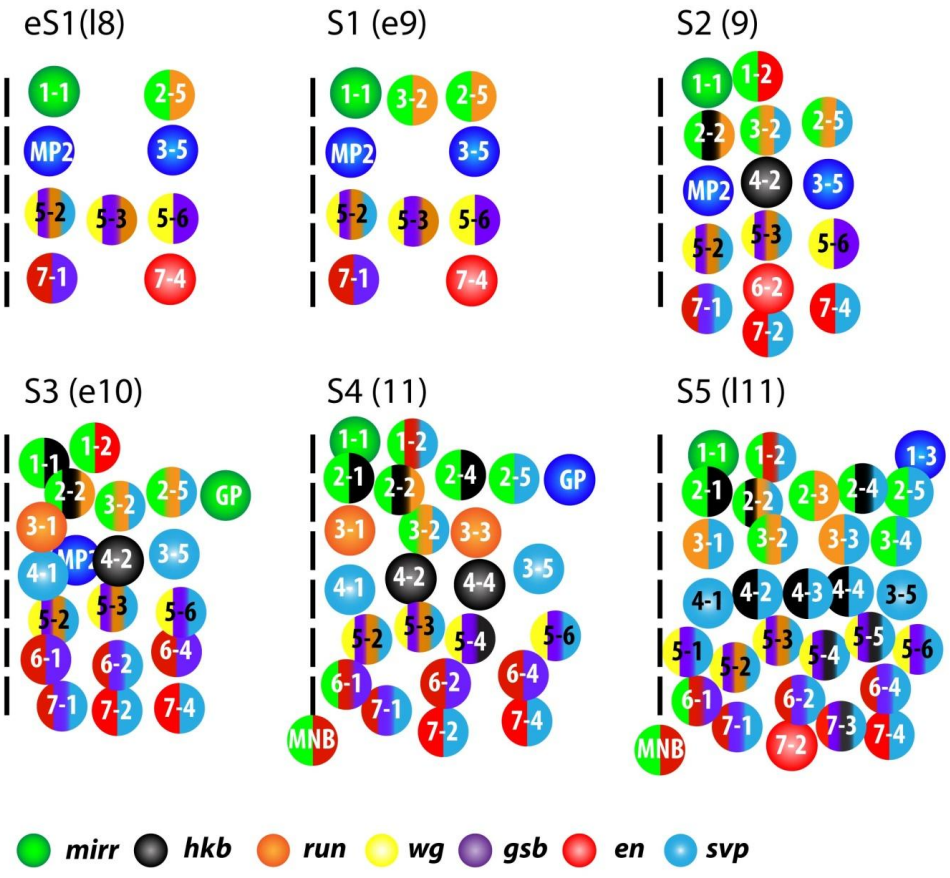

Figure 1-4: *Drosophila* neuroblast map

Figure 1-4: *Drosophila* neuroblast map

Neuroblasts in *Drosophila* delaminate in five segregation waves (S1 to S5). The neuroblast map shows the time of formation of each neuroblast and its gene expression pattern at the respective stage. The number in brackets gives the respective embryonic *Drosophila* stage (e stands for early, l for late, see appendix for *Drosophila melanogaster* staging). The midline is indicated by the dashed line. (eS1, S1) The first segregation wave S1 is divided into an early phase (eS1) with nine neuroblasts (NBs 1-1, 2-5, 3-5, 5-2, 5-3, 5-6, 7-1 and 7-4) and the midline precursor cell 2 (MP2) delaminating and the S1 phase where only one further neuroblast (NB 3-2) is formed. (S2) The next segregation wave S2 is characterized by the delamination of intermediate column neuroblasts 1-2, 4-2, 6-2 and 7-2 and the medial NB 2-2. (S3) During S3 three medial neuroblasts are formed (3-1, 4-1, 6-1), whilst laterally the glial precursor (GP) and NB 6-4 delaminate. Note that row 2, 3 and 4 neuroblasts are not yet arranged in their final row, whilst row five, six and seven neuroblasts form distinct rows. (S4) Neuroblasts 2-1, 6-1 and the median neuroblast (MNB) delaminate medially during S4. Additionally NBs 2-4, 4-2 and 5-4 delaminate. (S5) During the last segregation wave six more neuroblasts delaminate (1-3, 2-3, 3-4, 4-3, 5-1, 5-5 and 7-3). The lateral GP has disappeared. *engrailed* (*en*); *gooseberry* (*gsb*); *huckebein* (*hkb*), *mirror* (*mirr*); *runt* (*run*); *seven-up* (*svp*). Figure modified after Doe (1992) and Broadus *et al.* (1995).

Neuroblast identity

Every neuroblast is characterized by its position, differential gene expression, the fate of its progeny and the time of its formation (Fig. 1-4, Doe and Skeath 1996). This unique identity is obtained by spatial and temporal cues which partially already function in the neuroectoderm before neuroblasts are formed (Chu-LaGraff and Doe 1993, Skeath *et al.* 1995).

Spatial division of the neuroectoderm along the AP and DV axes is obtained by expression of a combination of segment polarity genes along the AP axis and columnar genes along the DV (also referred to as medio-lateral) axis (see Fig. 1-5; reviewed in Skeath 1999 and Bhat 1999). Expression of these genes ultimately divides the neuroectoderm in a Cartesian like coordinate system with each proneural cluster expressing a different set of segment polarity and columnar genes (Fig. 1-5) thereby enabling neuroblasts to obtain their unique identity.

Spatial identity of neuroblasts along the anterio-posterior axis

Segment polarity genes divide a segment into specific domains along the AP axis (reviewed in Peel *et al.* 2005). Although originally identified for their function during segmentation they have been demonstrated to regulate neuroblast formation and enable neuroblasts in different rows to obtain different identities (reviewed in Skeath 1999 and Bhat 1999). They are not only expressed in neuroectodermal cells but also in neuroblasts

and are therefore ideal candidates for neuroblast marker genes. Using several different segment polarity genes including *engrailed* (*en*), *wingless* (*wg*) and *gooseberry* (*gsb*), Doe (1992) identified and tracked individual neuroblasts throughout neuroblast formation. Figure 1-4 illustrates the expression of the genes discussed below over the five segregation waves (S1-S5) of neuroblast formation in *Drosophila*.

engrailed expression in cells of the nervous system of *Drosophila* was first described by DiNardo *et al.* (1985). All row six and seven neuroblasts, as well as NB 1-2, express *en* (Doe 1992). The expression begins with the formation of the neuroblast in the proneural cluster. Furthermore, some GMCs and neurons express *en*. The expression of *en* in the nervous system appears to be conserved between *Drosophila* and *Schistocerca* (Condron *et al.* 1994, Broadus and Doe 1995).

wingless is expressed in row five neuroectodermal cells and all row five neuroblasts (Doe 1992). *gooseberry* expression overlaps with *wg* expression in row five but expands onto neuroectodermal cells and neuroblasts of row six and NB 7-1 (Gutjahr *et al.* 1993). Proneural clusters and neuroblasts of row five depend on *gsb* expression to develop their unique identity. Loss of *gsb* transcripts results in proneural clusters of row five assuming the fate of row 3/4 proneural clusters. In contrast, the secreted Wg protein enables row three, four and six proneural clusters and neuroblasts to obtain their unique identity (Chu-LaGraff and Doe 1993, Bhat 1996, Bhat and Schedl 1997). Although *wg* is expressed in row five neuroblasts, these neuroblasts do not dependent on *wg* expression (Chu-LaGraff and Doe 1993).

A suitable marker for row one and two neuroblasts is *mirror* (*mirr*). *mirr* is expressed in a segmental pattern in the neuroectoderm out of which neuroblasts of row one and two delaminate (Broadus et al.1995). All row one and two neuroblasts express *mirr* from their formation until S5. Additionally NBs 3-2, 3-4, 6-1 and the MNB express *mirr* (Broadus *et al.* 1995).

Additional marker genes for neuroblast identity are, for example, the gap gene *huckebein* (*hkb*) and the pair-rule gene *runt* (*run*). These marker genes are not expressed in a strictly segmental manner in the neuroectoderm as is the case for the segment polarity genes

described above and their expression in the neuroectoderm is independent of their earlier function during segmentation (Doe 1992, Dormand and Brand 1998).

hkb is expressed in neuroblasts of row two, four and five and NB 7-3. In addition NB 1-1 expresses *hkb*, but although it is formed during S1 it only commences *hkb* expression once all S3 neuroblasts have formed. Therein NB 1-1 differs from all other *hkb* expressing neuroblasts which express *hkb* from their formation onwards. *hkb* is a useful marker to identify row four and row two neuroblasts. Furthermore, it is useful to distinguish row five neuroblasts, which all express *wg* but only NBs 5-4 and 5-5 express *hkb* (Doe 1992, Broadus *et al.* 1995, Chu-LaGraff *et al.* 1995). *hkb* mutants do not exhibit any change of identity. *hkb* expression is, however, crucial for correct axon pathfinding of several neurons (Chu-LaGraff 1995, Bossing et al 1996a) and for the formation of serotonin releasing neurons (Lundell *et al.* 1996).

The pair-rule gene *runt* is expressed in neuroblasts, GMCs and neurons (Kania *et al.* 1990, Duffy *et al.* 1991, Dormand and Brand 1998). Certain neuroblasts of row two, three and five express *runt* throughout neurogenesis (Dormand and Brand 1998). Furthermore, *runt* expression in NB 3-3 is required for *even-skipped* expression in the Eve lateral cluster (EL cluster; see Function and expression pattern of Even-skipped in *Drosophila* below) (Duffy *et al.* 1991, Dormand and Brand 1998)

Several other marker genes were used to establish the complete neuroblast map in *Drosophila*, including *eagle*, *fushi tarazu*, *odd-skipped* and *unplugged* (Doe 1992, Broadus *et al.* 1995). These are not dealt with in this work, however, and therefore are not discussed further.

In *Tribolium* several of the segmentation genes have been analysed for their role during segmentation (Sommer and Tautz 1993, Nagy and Carroll 1994, Brown *et al.* 1994a, b and c, Brown *et al.* 1997, Brown and Denell 1996, Choe *et al.* 2006, Farzana and Brown 2008, Choe and Brown 1999). However, hardly any data exist regarding their expression and function during neurogenesis. The established neuroblast map in the present work allowed thorough comparisons to be made between *Drosophila* and *Tribolium* regarding their expression in the nervous system.

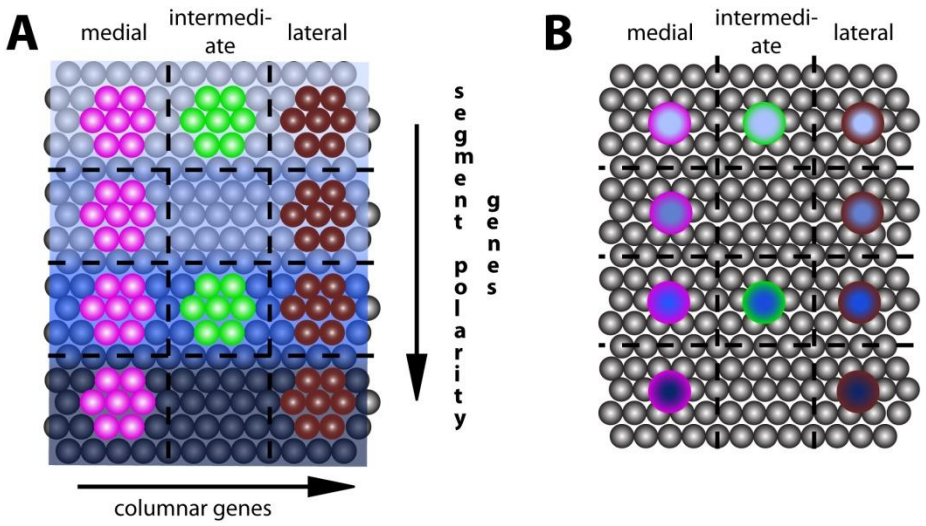

Figure 1-5: Spatial division of the neuroectoderm

One hemineuromere showing (A) the first proneural clusters out of which (B) S1 neuroblasts arise. (A) The expression of columnar genes (pink, green, brown) along the DV axis and segment polarity genes (blue) along the AP axis divides the neuroectoderm into a Cartesian like coordinate system. (B) Neuroblasts delaminate inside the embryo expressing the respective combination of segment polarity and columnar genes which have been expressed in the proneural cluster from which they originated in the neuroectoderm, thereby obtaining their unique identity.

Spatial identity of neuroblasts along the dorsal-ventral axis

The columnar genes (Skeath 1999) are involved in arranging the neuroectoderm along the dorsal-ventral axis, and divide the ventral neuroectoderm into three longitudinal columns in *Drosophila*. Originally four columnar genes were described - *ventral nerve cord defective* (*vnd*) expressed in the medial column, *intermediate neuroblast defective* (*ind*) in the middle column, *muscle segment homeobox* (*msh*) in the lateral column and the *epidermal growth factor receptor* (*EGFr*) expressed in the medial and intermediate column (Fig. 1-5; reviewed in Skeath 1999). Subsequently two more columnar genes – *Dichaete* and *Sox-Neuro* (*SoxN*) – functioning in parallel to *vnd* and *ind* were identified (Cremazy *et al.* 2000, Buescher *et al.* 2002, Overton *et al.* 2002, Zhao and Skeath 2002). The expression boundaries of the columnar genes along the dorsal-ventral axis coincide with the proneural clusters of S1 and S2. Interactions between the genes secure the establishment of different neuroblast fates along the DV axis (Skeath 1999).

The *Egfr* in *Drosophila* functions as a regulator of expression of the columnar genes and is expressed in the medial and intermediate column (Skeath 1998, Zhao *et al.* 2007). *Egfr* expression is required for the activation of *ind* expression and therefore for the formation of intermediate neuroblasts, but not necessary for maintaining its expression (Skeath 1998, von Ohlen and Doe 2000). The opposite is true in the case of *vnd*, for which activation of expression is independent of *Egfr* expression. Yet, *Egfr* is required to maintain *vnd* expression (Skeath 1998).

vnd is expressed in proneural clusters and neuroblasts along the medial column and later in development in GMCs and neurons (Jimenez *et al.* 1995, Mellerick and Nirenberg 1995) and is required for ventral neuroblast formation (Chu *et al.* 1998, McDonald *et al.* 1998). Furthermore, *vnd* inhibits *ind* expression in ventral column neuroectoderm (McDonald *et al.* 1998).

ind is expressed in proneural clusters and neuroblasts in the intermediate column and inhibits *msh* expression in the intermediate column (Weiss *et al.* 1998). Recent findings demonstrate the ability of *ind* to repress *vnd* expression and therein support the formation of the lateral border of *vnd* expression (Zhao *et al.* 2007). Intermediate neuroblasts are mostly dependent on *ind* expression similar to ventral neuroblasts and *vnd*.

msh is expressed in proneural clusters and neuroblasts in the lateral domain of the neuroectoderm (Lord *et al.* 1995, D'Alessio and Frasch 1996, Isshiki *et al.* 1997). In contrast to *Egfr, ind* and *vnd*, *msh* is not required for the formation of neuroblasts. Nevertheless, loss of *msh* results in alterations of the normal cell division pattern of dorsal neuroblasts and an aberrant migration pattern of neuronal progenitor cells, which ultimately results in disruptions of the commissure and connective pattern (Isshiki *et al.* 1997).

Wheeler *et al.* (2005) investigated the function of columnar genes in *Tribolium*, focusing on expression of four of the columnar genes (*Tc-vnd*, *Tc-ind*, *Tc-msh* and *Tc-Egfr*). In *Tribolium Tc-Egfr* appears to function in a similar way as in *Drosophila*, through the regulation of *Tc-ind* and *Tc-vnd* expression.

Tc-vnd expression in *Tribolium* resembles the expression of *vnd* in *Drosophila*. All neuroblasts delaminating out of the medial column express *Tc-vnd* for a brief period.

Furthermore, in *Tc-vnd* RNAi embryos 85% of ventral neuroblasts are lost and *Tc-ind* expression expands into the medial column with no change to number of intermediate or lateral column neuroblasts, which is consistent with the pattern observed in *Drosophila* (McDonald *et al.* 1998; Chu *et al.* 1998).

ind expression between *Drosophila* and *Tribolium* differs in the early expression in the neuroectoderm. In *Drosophila ind* is expressed along the entire neuroectoderm, whilst in *Tribolium* it is not expressed in the anterior part of the neuroectoderm. However, in both insects the expression gets restricted to one or two posterior neuroblasts by the end of germ band elongation (Weiss *et al.* 1998, Wheeler *et al.* 2005). Furthermore, as in *Drosophila* most intermediate neuroblasts are dependent on *Tc-ind* expression for their formation.

Wheeler *et al.* (2005) suggested that *Tc-msh* expression in *Tribolium* exhibits the most significant deviation from the expression of its orthologue in *Drosophila*. According to Wheeler *et al.* (2005) expression of *Tc-msh* in the neuroectoderm only begins midway through neurogenesis, in small clusters of neuroectodermal cells and neuroblasts within the lateral column. Subsequently, *Tc-msh* expression expands to more cell clusters and neuroblasts along the lateral domain until it is expressed in a continuous column along the lateral domain. Expression outside the neuroectoderm, in dorsal cells reaching from the lateral edge of the neuroectoderm to the edge of the germ band, commences after the onset of gastrulation and continues throughout germ band elongation. This is in stark contrast to *Drosophila* were *msh* expression in the neuroectoderm begins at stage five in small patches quickly forming a continuous band along the entire DV axis of the embryo. *msh* expression in the neuroectoderm of *Drosophila* is characterised by two phases of expression. The first period of *msh* expression lasts from expression in the dorsal neuroectoderm, prior to neuroblast formation, until S1 neuroblasts have delaminated. *msh* expression is re-initiated shortly before S3-S5 neuroblasts delaminate (Isshiki *et al.* 1997). Wheeler *et al.* (2005) therefore suggested that early-forming neuroblasts are independent of *Tc-msh* expression, whereas late-forming neuroblasts rely on *Tc-msh* to acquire lateral cell fate, as is the case in *Drosophila*.

Spatial identity of neural precursor cells in arthropods other than insects

Investigations in arthropods other than insects have revealed similar gene expression pattern of segmentation and columnar genes. Engrailed expression, for example, appears to be conserved not only between insects and crustaceans but amongst all arthropods studied (Patel 1994, Patel *et al.* 1989, Duman-Scheel and Patel 1999, Chipman and Stollewerk 2006, Fabritius-Vilpoux *et al.* 2008). Additionally the columnar genes are also expressed in three domains in *Cupiennius salei* (Chelicerata; Arachnida (spider)) and *Glomeris marginata* (Myriapoda; Diplopoda, (millipede)) (Dove 2003, Doeffinger and Stollewerk 2010). There are, however, noticeable differences in the expression pattern of *msh* between *Drosophila* and the spider *Cupiennius*. In *Cupiennius* all NPGs forming in the lateral *msh* domain continue *msh* expression throughout neurogenesis whereas in *Drosophila* one third of neuroblasts delaminating out of the msh^{+} lateral domain do not express *msh* (Doeffinger and Stollewerk 2010). The data obtained in chelicerates and myriapods suggest that the stereotyped arrangement of neuroblasts and groups of neural precursor cells in arthropods (see Fig. 1-3) may be achieved by a conserved function of the segment polarity genes and columnar genes described above (Doeffinger and Stollewerk 2010).

Temporal identity of neuroblasts

The previous paragraphs discussed in detail the mechanisms by which a neuroblast obtains its positional identity. This, however, does not explain how the same neuroblast generates neural progenitor cells of different identities. Previous work has demonstrated that this is achieved by temporal changes in gene expression. Most neuroblasts express a set of four genes in a temporally sequential manner (reviewed in Brody and Odenwald 2002, 2005, Pearson and Doe 2004). These temporal genes, in order of their occurrence in neuroblasts, are *hunchback* (*hb*)*, Krüppel* (*Kr*)*, nubbin* (*nub*; also called *pdm-1*) and *castor* (*cas*; *also* called *ming*), with the GMCs maintaining the respective expression the neuroblasts had at their time of formation. The expression of a specific temporal gene by a GMC is then passed on to the neurons or glial cells formed. Hence *hb* and *Kr* are expressed in early-born deep layer neurons, *nub* in middle layer neurons and *cas* in late-born superficial layer neurons (see figure 1-6, Cui and Doe 1992, Mellerick *et al.* 1992, Kambadur *et al.* 1998, Isshiki *et al.* 2001, Novotny *et al.* 2002, Cleary and Doe 2006, Grosskortenhaus *et al.* 2006).

Selected early and late formed neuroblasts have been studied in detail, revealing that not all neuroblasts express the complete sequence of four temporal genes (Isshiki *et al.* 2001). Additionally, many neuroblasts commence the expression of a further gene called *grainy head* (see figure 1-6; Brody and Odenwald 2000). These temporal genes do not, however, regulate cell-type identity itself but rather the differentiation into early and late born neural progenies. For example, the first GMC formed by NB 6-4 expresses *hb* but ultimately generates glial cells, whereas NB 7-4 generates glial cells only later in development which are not hb^+ (Isshiki *et al.* 2001). It should be noted that different neuroblasts in a given hemisegment may express different temporal genes at the same time. For example an early-formed neuroblast may already have gone through the entire gene expression cascade, generating *cas* expressing neurons, whilst a late-formed neuroblast in the same hemisegment is generating *hb* or *Kr* expressing neurons (Isshiki *et al.* 2001). The temporal gene expression cascade functions in isolated neuroblasts in cell culture, showing that no interaction between neuroblasts and cells of the neuroectoderm is necessary to ensure proper lineage development (Brody and Odenwald 2000). Temporal genes appear to intrinsically interact with each other, regulating the transition

from expression of one gene to the other by activating or inhibiting each other in a neuroblast (Brody and Odenwald 2002, 2005; Pearson and Doe 2004). It has, however, been demonstrated that inactivation of early temporal genes (*e.g. hb*) does not alter expression of later temporal genes (*e.g. cas*) (Kambadur *et al.* 1998, Brody and Odenwald 2000, Isshiki *et al.* 2001). Therefore additional genes are likely to be involved in specifying temporal identity to neural progenitor cells.

One such gene, which was previously used as a neuroblast marker, is *seven-up*, which is expressed in all neuroblasts but four (Fig. 1-4; Doe 1992). Doe (1992) used *seven-up* as a marker to follow specific neuroblasts throughout neurogenesis. Some neuroblasts (*e.g.* NB 5-2) express it from the time of their formation until the last stage of neuroblast formation, whilst others only start expressing *seven-up* long after they have formed and already generated several GMCs (*e.g.* NB 3-1). This temporally restricted expression pattern indicates a function in the temporal gene cascade. Indeed, Kanai *et al.* (2005) demonstrated the role of *seven-up* in generating neuronal diversity by enabling the switching of *hb* to *Kr* expression. Loss of *seven-up* generated more early born neurons, reflecting prolonged *hb* expression. In contrast to the temporal genes, *svp* expression is not detected in neurons (Kanai *et al.* 2005).

No data regarding temporal gene expression in insects other than *Drosophila* are available. Therefore one aim of the present work was to analyse their expression and draw conclusions on their function during neuroblast differentiation in *Tribolium*.

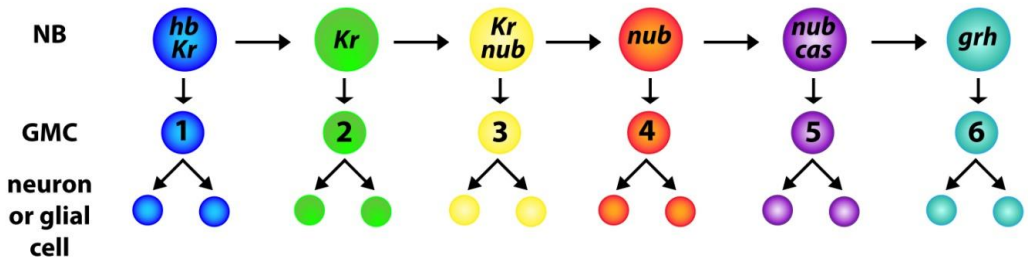

Figure 1.6: Temporal gene expression in *Drosophila* neuroblasts

Temporal mechanisms determine the identity of a neuroblast and its progenitor cells after delamination. Every neuroblast sequentially expresses a set of temporal patterning genes (*hb*, *Kr*, *nub*, *cas*, *grh*) passing on the respective expression to its daughter cells (GMC, neurons and/or glial cell) and thereby defining their identity at a specific point in time. Not every neuroblast, however, expresses the whole set of temporal genes (see text). Neuroblast (NB), ganglion mother cell (GMC). Figure modified after Pearson and Doe (2004).

Neuron formation in *Drosophila*

In *Drosophila* 30 neuroblasts of a hemisegment generate around 370 neural cells which can be subdivided into around 310 interneurons, 32 motor neurons (38 in thoracic hemisegment), 7 neurosecretory cells and 30 glial cells (Ito *et al.* 1995, Landgraf *et al.* 1997, Schmid *et al.* 1999). Neuroblast lineages have been analysed and almost all neurons and glial cells can be assigned to their origin. Together the intermediate and ventral neuroblasts of one hemisegment produce around 200 neurons and only three glial cells, whereas lateral neuroblasts only produce around 120 neurons but around 27 glial cells (Bossing *et al.* 1996b, Schmidt *et al.* 1997, Schmid *et al.* 1999).

The axonal scaffold of insects develops from a group of early neurons, called pioneer neurons (Bate 1976), which grow along stereotypic routes establishing the primary axonal tracts (Thomas *et al.* 1984). Longitudinal connectives join the single segments and transversal commissures connect the two hemineuromeres of a segment (Fig. 1-7). Neurons are subdivided into motor neurons and interneurons. Motor neurons innervate body wall muscles and project their axons towards these muscles via two nerves, the intersegmental nerve (ISN) and the segmental nerve (SN). Furthermore, the segmental nerve consists of four branches (SNa, SNb, SNc, SNd) (reviewed in Landgraf 2006). In contrast, interneurons are neurons that connect different neurons.

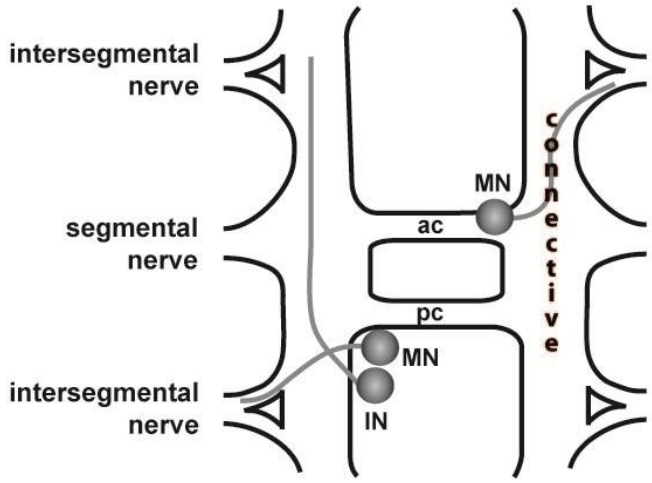

Figure 1-7: Illustration of ventral nerve cord in *Drosophila*

Anterior commissure (ac), posterior commissure (pc), motor neuron (MN), interneuron (IN); modified after Linne (2010).

Neural marker gene expression in differentiating neural cells

Several marker genes including members of the lim homeodomain proteins *apterous* (*ap*), *islet* (*isl*), lim3, the homeodomain protein dHb9 (also known as *extra-extra*) and *even-skipped* are expressed in distinct subsets of neurons, thus allowing their identification (Doe *et al.* 1988, Lundgren *et al.* 1995, Thor *et al.* 1999, Thor and Thomas 1997, Landgraf *et al.* 1999, Broihier and Skeath 2002). *ap* is an example of a neuron specific gene which is expressed in seven interneurons (Lundgren *et al.* 1995). All *ap* expressing interneurons form a single nerve bundle. Furthermore, *ap* expression does not overlap with *isl* expression (Thor and Thomas 1997). A further example of a neuron specific marker is *lim3* (Thor *et al.* 1999) which is not co-expressed with *isl* or *ap* in interneurons. Co-expression with *isl* is, however, detected in some ISN specific motorneurons. dHb9 is co-expressed with *ap*, *isl*, *eve* and *lim3* in several motor- and interneurons (Broihier and Skeath 2002).

This thesis focused on the expression pattern of *even-skipped* and *islet* (known as *tailup* (*tup*) in *Tribolium*) in *Tribolium*. Therefore a detailed description of their respective expression pattern and function in *Drosophila* is provided in the following paragraphs.

Function and expression pattern of *even-skipped* in *Drosophila*

The pair rule gene *eve* is expressed in motorneurons with targets in dorsal muscle fields (Landgraf *et al.* 1999). Loss of *eve* expression disrupts correct truncation of the intersegmental nerve and prevents it from reaching dorsal parts of the muscle field. As is the case for those genes described above, *eve* is expressed in GMCs and neurons (Frasch *et al.* 1987, Doe *et al.* 1988). There are around 16 neurons per hemineuromere which express *eve* (Fig. 1-8; Doe *et al.* 1988, Patel *et al.* 1989, Broadus *et al.* 1995). NB 1-1 generates the anterior and posterior corner cells (aCC motor neuron/pCC interneuron) which constitute two of the pioneer neurons. The aCC motor neuron and the pCC interneuron form a seven cell cluster with the U/CQ neurons which are formed by NB 7-1 (Broadus *et al.* 1995, Bossing *et al.* 1996b, Landgraf *et al.* 1999). Furthermore, Broadus *et al.* (1995) describe a cell positioned close to pCC termed the friend of pCC (fpCC) which is also formed by NB 7-1. Anterior to the aCC and pCC neurons the RP2 neurons, generated by the first GMC of NB 4-2, express *eve* on either side of the midline. A further

eight to ten neurons are formed by the lateral neuroblast 3-3 (Schmidt *et al.* 1997). As they are positioned laterally in the hemineuromere they are termed the Eve lateral cluster (EL cluster) (Patel *et al.* 1989). Investigations of the expression pattern of *even-skipped* in other insects and several species of crustaceans have revealed that the overall expression pattern is conserved in the above named neurons between hexapods and crustaceans (Duman-Scheel and Patel 1999, Patel 1992).

The expression of *eve* in a distinct subset of neurons allowed it to become a marker with which to analyse cell fate changes in the development of the nervous system. In *Drosophila* it was demonstrated that the U/CQ neurons, aCC/pCC neurons are missing in *vnd* mutants whereas the RP2 neuron is sometimes duplicated suggesting failure of ventral neuroblast formation and identity changes (Chu *et al.* 1998, McDonald *et al.* 1998, Mellerick and Modica 2002).

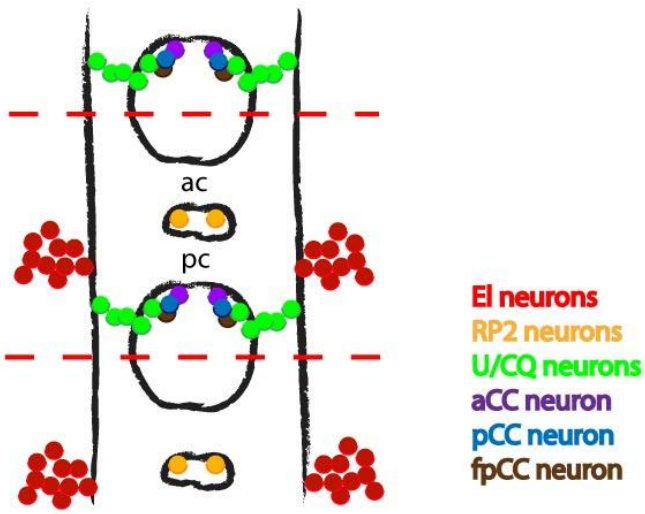

Figure 1-8: Eve+ neurons in *Drosophila*

Illustration of neurons that express Even-skipped in the *Drosophila* nervous system. The black lines indicate the axonal scaffold; the segment borders are indicated by the red dashed lines; anterior commissure (ac); posterior commissure (pc). Illustration modified after Broadus *et al.* (1995) and Mellerick and Modica (2002).

tailup is expressed in motor- and interneurons in *Drosophila*

tailup (also referred to as *islet*) belongs to a sub-family of LIM homeodomain genes and is initially expressed in precursor cells of the heart, aorta, pharynx and amnioserosa. In the nervous system it is expressed in 20 to 30 motorneurons and interneurons (Thor and Thomas 1997, Broihier and Skeath 2002). Motorneurons which are *tup* positive are, amongst others, the RP1, 3 and 4 neurons, generated by NB 3-1 (Landgraf *et al.* 1997). Most of the *tup* expressing motorneurons exit the ventral nerve cord via two branches of the segmental nerve (SNb and SNd) and innervate ventrally located muscles. Furthermore, *tup* expression is found in multiple interneurons of which a subset constitute the 3 dopaminergic and 4 serotonergic interneurons per segment (Lundell and Hirsch 1994, Thor and Thomas 1997). Neuroblast 7-3 forms the only two serotonergic interneurons per hemisegment (Lundell *et al.* 1996). Of the three dopaminergic cells per segment only the origin of the H-cell, which is generated by midline progenitor cell 3 (MP3), has been proven (Goodman *et al.* 1981, Bossing and Technau 1994). The other two dopaminergic interneurons are likely to be progeny of NBs 5-1 and 5-6 (Tio *et al.* 2011). Loss of function studies have demonstrated that *tup* expression is not necessary for the formation of neurons but vital for correct axon pathfinding and expression of the neurotransmitters dopamine and serotonin (Thor and Thomas 1997).

Studies of expression of the neuronal marker genes *eve* and *tup* have revealed further differences between neural specification in arthropods (Doeffinger and Stollewerk 2010). In *Cupiennius tup* expressing neural progeny are generated by laterally positioned *msh* expressing NPGs, whilst in *Drosophila* tup^{+} neurons are formed by ventral and medial NBs. Furthermore, *eve* is only expressed in two NPGs in *Cupiennius* and never coincides with *tup* expression. In *Drosophila*, in contrast, several neuroblasts generate eve^{+} neurons of which two co-express *eve* and *tup*. In summary, it appears that the conserved arrangement of neuroblasts and neural precursor groups in insects and chelicerates is obtained by partially conserved expression of segment polarity genes and columnar genes in the neuroectoderm. Conspicuous changes are observed, however, in the identity of neural progenitor cells (Doeffinger and Stollewerk 2010).

Aims

In the last thirty years great knowledge on early neurogenesis in insects, with a special focus on *Drosophila melanogaster,* has been obtained. Species of different groups of insects (silverfish, grasshopper, stick insect and flies) have been studied, mostly on a morphological level. Molecular data outside *Drosophila* is rare. Recent studies have investigated early mechanisms involved in neuroblast formation and the division of the neuroectoderm into three distinct domains (medial, intermediate and lateral column) in the beetle *Tribolium castaneum*, thereby establishing *Tribolium* as a Coleopteran model for further studies in neurogenesis.

The aim of the work presented here was to provide a detailed comparison of early neurogenesis between two relatively closely related holometabolous insect species in order to understand how, and at what level, modifications of the nervous system manifest themselves. Initially the formation and arrangement of neuroblasts was analysed, resulting in the creation of a neuroblast map for *Tribolium*. Subsequently, changes in neuroblast identity between *Tribolium* and *Drosophila* were investigated, by performing gene expression studies on segmentation and columnar genes that are known to establish neuroblast identity in *Drosophila*. Finally, the impact these changes in gene expression expression may have on the formation and differentiation of neural progeny were analysed by performing RNA mediated interference studies. In order to visualise differences in differentiation of neurons the expression pattern of two neuron specific marker genes - *even-skipped* and *tailup* - were analysed in wild type and RNAi embryos.

2 Materials and Methods

Chemicals and solutions

Chemicals were obtained from VWR (UK) unless otherwise mentioned. Recipes for solutions are listed in the appendix.

Animal handling

Tribolium castaneum stock

A culture of *Tribolium castaneum* was established using the wild type strain “San Bernardino” kindly provided by Max Telford’s lab (UCL London). *Tribolium* were maintained on wholemeal flour supplemented with 5% yeast at 28°C and around 40% humidity. To minimize potential biological contaminants the flour and yeast mixture was frozen at -20°C for 24 hours before use. The flour was changed every two to three days and the old flour with eggs in it, was used to establish new beetle stocks.

Embryo collection

Prior to egg collection the beetles were transferred to plain white flour and incubated at 32°C for one hour. The adult beetles were separated from the white flour, larvae, pupae and eggs using a sieve with a mesh size of 800 µm. To separate the larvae and pupae from the flour and eggs a sieve with a mesh size of 500 µm was used. Finally, separation of the eggs was achieved by passing the flour through a sieve with a mesh size of 250 µm. The collected eggs were kept on a petri dish at 32°C until further usage.

Dechorionisation, fixation and devitellinisation of embryos

Prior to fixation of the embryos the chorion was removed. The embryos were transferred from the petri dish into a *Drosophila* mesh basket and rinsed with tap water. They were then incubated twice for three minutes in 50% chlorine bleach solution and afterwards thoroughly washed with tap water. Using a small spatula the dechorionated embryos were transferred to a 100 ml glass bottle containing a biphasic solution of 3 volumes heptane

and 1 volume 4% formaldehyde in PEMS solution. The embryos were fixed on a shaker at 200 rpm for 25 min. Afterwards the soluble phase (PEMS) was replaced by 8 ml cold methanol. The embryos were then shaken vigorously for around 30 seconds. Devitellinised embryos sank to the bottom of the vessel and were transferred to methanol using a plastic pipette. The remaining embryos were washed several times in methanol to remove any residues of heptane and manually devitellinised under a stereo microscope, using fine tungsten needles. Devitellinised embryos were stored at -20°C in methanol and used for *in situ* hybridisation and antibody staining.

For phalloidin staining the protocol differed in some respects. Instead of using formaldehyde, as mentioned above, which contains traces of methanol, methanol free formaldehyde (Polysciences, Europe) was used for fixation. The embryos were fixed for one hour, before they were transferred to ethanol and manually devitellinised. Devitellinised embryos were fixed again in 1 ml PBTriton (PBS + 0.1% Triton-X-100) and 140 µl methanol free formaldehyde overnight.

Immunohistochemistry

Phalloidin staining

Freshly fixed embryos, as described above, were transferred from 100% ethanol to 100% PBTriton via a 25%, 50% and 75% ethanol/PBTriton series. After several washing steps in PBTriton (twice for 5 min followed by three times for 10 min) they were blocked in PBTriton + 1% BSA at room temperature (RT) for one hour. Meanwhile Alexa Fluor® 488 phalloidin (Invitrogen, UK) was diluted 1:10 in PBTriton. The embryos were incubated with the phalloidin solution between 24h and 48h in the dark at 4°C. To stop the reaction the phalloidin solution was removed and the embryos were washed several times in PBS (three times for 5 min, followed by three times for 20 min). Before the embryos were transferred to 70% glycerol in PBS a Hoechst cell nuclei staining was performed (see Nuclei Stainings section, below). Afterwards, the embryos were dissected under a stereo microscope and analysed using confocal microscopy.

Antibody staining

Fixed embryos were transferred via a methanol PBTween series into 100% PBTween (PBS + 0.02% Tween-20). After several washes in PBTween (a single rinse followed by three 20 minute washes) and a one hour blocking step in blocking reagent (Perkin Elmer) at RT, the primary antibody diluted in PBTween was incubated for 4h at RT or overnight at 4°C. The reaction was stopped by several washing steps in PBTween (rinse once, wash for 10 min, followed by a further two washes for 20 min). Finally, the secondary antibody, diluted in PBTween, was incubated for 2h. The following procedure varied, depending on the antibody used. When using a fluorescent secondary antibody the incubation and all the following steps were performed in the dark. The embryos were washed several times in PBTween and were then transferred to glycerol PBS. If a colorimetric reaction was used to detect the secondary antibody the embryos were washed several times in PBTween (rinse twice, wash for 10 min, wash twice for 20 min followed by a final wash of 30 min) before the staining took place (see NBT/BCIP or Fast Red staining).

Table 2.1: Primary antibodies

Antibody	Species	Dilution	Supplier
α-tubulin	Mouse	1:100	Sigma
α-engrailed (4D9)	Mouse	1:20	DSHB
α-even-skipped (3C10)	Mouse	1:20	DSHB
α-islet1 (40.3A4)	Mouse	1:20	DSHB
α-horse radish peroxidase (HRP)	Rabbit	1:250	DSHB
α-Dig-alkaline phosphatase (AP)	Sheep	1:2000	Roche
α-fluorescein-AP	Sheep	1:1000	Roche
α-Dig-peroxidase	Sheep	1:500	Roche

Table 2.2: Secondary antibodies

Antibody	Label	Species	Dilution	Supplier
anti-mouse	Cy3	donkey	1:1000	JIR
anti-mouse	AP	donkey	1:1000	JIR
anti-rabbit	Cy3	donkey	1:1000	JIR

Colorimetric staining

To visualise protein or mRNA expression after antibody staining and/or *in situ* hybridisation colorimetric stainings were performed. Embryos were transferred to staining dishes and kept in the dark. The staining reaction was checked at regular intervals. Depending on the probe or antibody used, the staining duration differed from 1h up to 2 days. To stop the reaction the embryos were rinsed and washed several times in PBTween (rinse twice, wash three times for 5 min prior to a final wash for 15 min) and stored in 70% Glycerol/PBS at 4˚C.

NBT/BCIP staining

Embryos were transferred to a staining dish and washed three times for five minutes in AP-buffer (pH 9.5), before the staining solution containing 3.5 µl BCIP (5-Bromo-4-chloro-3-indolyl phosphate; Sigma-Aldrich, UK) and 4.5 µl NBT (Nitro blue tetrazolium; Sigma-Aldrich, UK) in 1 ml alkaline phosphatase (AP)-buffer was added.

Fast Red staining

For Fast Red staining ready-made tablets from Sigma-Aldrich (UK) were used. One tablet of Tris buffer pH 8.2 was dissolved in 1 ml H_2O by vortexing. Subsequently, a Fast Red tablet was added and the mixture was vortexed again. The final solution was centrifuged for 5 min at 10,000 rpm resulting in the formation of a red pellet at the bottom of the tube. The staining solution was added to the embryos after washing them three times for 5 minutes each in Tris buffer.

Nuclei staining

Hoechst and SYTOX® Green staining

To visualise the cell nuclei either Hoechst or SYTOX® Green (Invitrogen/Life Technologies, UK) was used. After antibody staining, *in situ* hybridisation or phalloidin staining, the embryos were washed several times in PBTween and then incubated in Hoechst (1:1000 in PBTween) for 10 min or SYTOX® Green (1:1000 in PBTween) for 1h. Afterwards they were washed several times and transferred to 70% Glycerol/PBS and stored at 4°C.

In situ hybridisation

Single probe *in situ* hybridisation

For *in situ* hybridisation, embryo baskets or 1.5 ml micro-centrifuge tubes were used. The devitellinised embryos were re-hydrated from 100% methanol to 100% PBTween. They were washed twice in 100% PBTween before they were pre-hybridised with pre-warmed hybridisation solution at 60°C for one hour. Three microlitres of probe were added to 1 ml hybridisation solution and denatured at 85°C for 10 min. The hybridisation and probe solution replaced the hybridisation solution and the embryos were incubated overnight at 60°C in a water bath. The next day the embryos were washed for 15 minutes in 50% formamide in 2x SSC, followed by a second 60 minute wash step. They were then incubated for 15 minutes in 2x SSC buffer and 0.1% Tween-20 followed by three 15 minute steps in 0.2x SSC buffer and 0.1% Tween-20. All steps were carried out at 60°C. From then on, all steps were carried out at RT. The embryos were washed three times for five minutes with PBTween before they were then blocked for one hour in blocking buffer (Perkin Elmer). The primary antibody (either anti-DIG-AP or anti-fluorescein-AP) was incubated at a dilution of 1:2000 in blocking buffer for two hours at RT or overnight at 4°C or for 2h at RT. To wash unspecifically bound antibody out of the tissue, the embryos were washed five times for 20 minutes each in PBTween or twice for 20 minutes and then overnight at 4°C. Colorimetric and nuclei staining followed, as described above.

Fluorescent *in situ* hybridisation

The protocol for fluorescent *in situ* hybridisation differed from that described above in the use of anti-DIG-peroxidase (POD) or anti-fluorescein-POD at a dilution of 1:500. The TSA™PLUS Fluorescence Kit (Perkin Elmer, UK) was used to amplify the signal. After two hours incubation with anti-DIG-Pod and five 20 minute washes in PBTween, 250 µl of “Plus Amplification Diluent” was added to the embryos. The diluent was replaced by a mixture of 96 µl “Plus Amplification Diluent” and 4 µl staining solution five to ten minutes later. After two hours incubation, several washing steps followed, to minimise background staining. Nuclei staining followed as described above.

Molecular methods

Isolation of total RNA

Two-hundred and fifty microlitres of a mixture of embryos of different stages, which were kept at -80°C, were homogenised in 750 µl TRIZOL and incubated for five minutes at RT. After adding 200 µl of chloroform the tube was shaken vigorously by hand and left for three minutes at RT. The mixture was then centrifuged for 15 min at 13000 rpm and 4°C. The supernatant (aqueous phase) was transferred to a new 1.5 ml tube and the RNA was precipitated by mixing it with 500 µl 100% isopropanol and allowing it to incubate for 10 min at RT. To collect the precipitated RNA, the tube was centrifuged for 15 min at 13000 rpm at 4°C and the supernatant was removed on ice. The pellet was washed with 500 µl cold 70% ethanol and thoroughly re-suspended before it was again centrifuged for five minutes at 7500 rpm and 4°C. After removing the supernatant, the pellet was allowed to dry for approximately two minutes. Finally, the RNA was dissolved in 50 µl DEPC-treated H_2O and incubated for 10 minutes at 60°C. To check the quality of the resulting RNA, one microlitre was loaded onto an agarose gel. Additionally, the RNA concentration was measured using a Nanodrop spectrophotometer (NanoVue™, Version 4282V1.7.3, GE HEALTHCARE). The remaining RNA was stored at - 80°C.

Reverse transcription (cDNA synthesis)

cDNA was generated using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, UK). Three microlitres of RNA (up to 5 µg total RNA), 1 µl Oligo (dT) primer (50 µM), 1 µl dNTPs (10 mM) and 5 µl DEPC - H_2O were combined in a 1.5 ml tube and incubated for 5 min at 65°C. Meanwhile, a reaction mixture consisting of 2 µl 10x RT-buffer, 4 µl $MgCl_2$ (25 mM), 2 µl DTT (0.1 M), 1 µl RNase out (40 u) and 1 µl SuperScript III (200 u) was set up. The primer/RNA mixture was cooled to 4°C (for approximately 1 minute) before the reaction mixture was added. The reaction was incubated for 50 min at 50°C followed by a 5 min incubation step at 85°C. The reaction was chilled on ice and collected by brief centrifugation. Finally, 1 µl RNase H (2 u) was added and a final incubation for 20 min at 37°C followed. The generated cDNA was kept at - 20°C.

Primer sequences

Primer sequences were identified using the freely available Primer3 software (primer3.sourceforge.net). The previously sequenced genome of *Tribolium* allowed to generate specific primers for all genes of interest. In some cases a single pair of primers was sufficient to amplify the desired target sequence. Where this was not the case nested primers were employed. Table 2.3 gives details of all primer sequences used. With the exception of the M13 primers (17bp), *asense* forward (19bp), *asense* nested reverse (18bp), *ind* reverse (22bp) and *vnd* nested forward (18bp) all primers consisted of 20bp. Primers were ordered from Sigma Aldrich Ltd UK. Tm in Table 2.3 gives the melting temperature supplied by Sigma.

Table 2.3 Primer sequences to generate single stranded RNA probes forward (for); reverse (rev)

Gene	Orientation	Sequence (5'-3')	Tm in C°
M13	**for** **rev**	**CTGGCCGTCGTTTTACA** **CAGGAAACAGCTATGAC**	**50.70** **57.60**
***asense* (*ase*)**	**For**	**TAGGGCCAATTTTCCGAGA**	**64.3**
	Rev	**AGGGTATCGAGGAGGTGGAG**	**64.3**
	nested for	**CGACTTCCGAATTTGTGTGA**	**63.7**
	nested rev	**AACGGTGCTTTCGTCGAG**	**64.1**
***castor* (*cas*)**	**For**	**CCAAAACAGCAGCACTTCAA**	**64**
	Rev	**AAGGTGTAGCGGATGGTTTG**	**63.8**
***engrailed* (*en*)**	**For**	**AGACACTCCGCTCTCCTTCA**	**64.2**
	Rev	**AGACACTCCGCTCTCCTTCA**	**63.8**
***gooseberry* (*gsb*)**	**For**	**GGACGTCCTTTACCGAATCA**	**63.8**
	Rev	**TGAGGAAGAGCTCCACTGCT**	**64.3**
	nested for	**GCATAAGACCCTGCGTGATT**	**63.9**
	nested rev	**GAAAAGCCGACGGTAGTGAC**	**63.6**
***huckebein* (*hkb*)**	**For**	**CCCTTCCTGCAATAACGAAA**	**63.7**
	Rev	**ACACTCCTCTTGGCTGGAAA**	**63.8**

***hunchback* (*hb*)**	**for**	**AGCACCACCTGGAGTACCAC**	**64.1**
	rev	**CTCCTTCTTCGCCTCCTCTT**	**63.8**
***Intermediate neuroblast defective* (*ind*)**	**for**	**ATTCCCTCATCAGCAACACC**	**63.9**
	rev	**CCGAAAAATCTAATGACAATGG**	**62**
	nested for	**CCAAAGACATGAACGCAAGA**	**63.8**
	nested rev	**AACAGCATTTGTTGCCATTG**	**63.5**
***Krüppel* (*Kr*)**	**for**	**TTATCGTCGGTCTCGTACCC**	**63.8**
	rev	**CCGCAAAATTCCACAAAACT**	**63.6**
	nested for	**AACGAAAAACGTGGAAGGTG**	**63.8**
	nested rev	**TCTGGAGGAACTCGCATACC**	**64.2**
***mirror* (*mirr*)**	**for**	**GTATCCAACCAAAGGCGAAA**	**63.6**
	rev	**GCCGATGGCAGTCACTTTAT**	**63.9**
	nested for	**GACGACCACAAGAGCACAGA**	**64.3**
	nested rev	**GCCTCTAGAGACGGCTTCAA**	**63.5**
***muscle segment homeobox* (*msh*)**	**for**	**AAGATCTCCTTCAGCGTGGA**	**63.9**
	rev	**GGGGTATGTTGAGGATGTGC**	**64.2**
***nubbin* (*nub*)**	**for**	**AAACCAGGTCCAACAAATCG**	**63.6**
	rev	**CTTCTCCATGCAGAGGCCGT**	**68.9**
***prospero* (*pros*)**	**for**	**ACGACCAATAACGCTGGAAC**	**63.8**
	rev	**GATTGGTTCCGCACTTTCAT**	**63.8**
	nested for	**TGAGCCGCGTAACTCCTACT**	**63.8**
	nested rev	**GTTTCTTGCAGCCGTTGACT**	**64.3**
***runt* (*run*)**	**for**	**GCGGTGATGAAGAACCAAGT**	**64**
	rev	**TCTTGCACAGTGGAGTCAGG**	**64.3**
	nested for	**AGTTCAACGATTTGCGGTTC**	**63.9**
	nested rev	**AAGGTTGGCAGGTTTGATTG**	**63.8**
***seven-up* (*svp*)**	**for**	**TATCGACCAACACCACAGGA**	**64.1**
	rev	**TGTCCGTAAACTGGGGAGTC**	**63.9**
***tailup* (*tup*)**	**for**	**TAAGTTCGTTCGTCGGCTGT**	**64.6**
	rev	**GCTGATGGGGTTGCTCTAAG**	**64**

	nested for	**GGACTCGCAGAACAAAAAGC**	**63.9**
	nested rev	**GCTGATGGGGTTGCTCTAAG**	**63.9**
***ventral nervous system defective* (*vnd*)**	**For**	**TTACACTCCCATCACCAGCA**	**64.2**
	Rev	**GAACATCGGCCACTGAATTT**	**63.8**
	nested for	**CCATCCTGCCCAACTCAG**	**64.5**
	nested rev	**TGACCTGACCGAAACAACAA**	**64.2**
wingless (*wg*)	**For**	**CCGAACAATCTATCGCCAAT**	**63.6**
	Rev	**GGTTTCAACTGGAAGCCGTA**	**63.9**
	nested for	**AGGTGCCAATAATGCGATTC**	**63.7**
	nested rev	**CGGCGATTCTCCCTCTTACT**	**64.4**

Primer for RNAi interference

In order to generate double stranded *Tc-vnd* and *Tc-ind* RNA, the primers used for the generation of RNA probes stated in Table 2.3 were employed. In the case of *Tc-msh* several additional pairs of primers were evaluated. Initially the primers described in Table 2.3 were tested, followed by three further primer pairs designed to amplify different regions of the *T. castaneum msh* gene (see Table 2.4).

Table 2.4 Primer sequences for double stranded *msh* RNA

Orientation	Sequence	Tm in C°	length	product size
forward 1	**CGAAAGCCGCGCACCCCCTTC**	**78.9**	**21**	**195bp**
reverse 1	**CTTCTCCAGCTCGGCCTCCTG**	**71.6**	**21**	
forward 2	**AAGATCTCCTTCAGCGTGGA**	**63.9**	**20**	**170bp**
reverse 2	**TGCGGTATTAAAACGGTGTG**	**63.2**	**20**	
forward 3	**GCTTTGCACGGTTTCAGTCC**	**66.9**	**20**	**870bp**
reverse 3	**ACCTTTGTACATCATAAACAAACACA**	**62.1**	**26**	

PCR reaction

PCR reactions were carried out in a total volume of 10 µl, mixing 0.5 µl cDNA, 1 µl dNTPs (2 mM, Sigma-Aldrich, UK), 1 µl Thermo pol Buffer (10x, New England Biolabs, UK), 0.5 µl forward primer (10 mM), 0.5 µl reverse primer (10 mM), 0.1 µl Taq polymerase (2.5 u, New England Biolabs, UK) and 6.4 µl ddH_2O. If there were no or very weak bands visible after the first PCR, a second PCR, using the same primer pair or a PCR using nested primers was performed using the primary PCR-product as a template.

All PCRs were performed on a TECHNE TC-512 Thermal Cycler (Bibby Scientific Limited, UK), with the lid heated to 105°C. The following thermal cycle was used:

5 min 94°C

30 sec 94°C

1 min annealing temperature ($T_A = T_M - 2$)

60 or 90 sec elongation time (depending on the length of the fragment) 72°C

repeat steps two to four 32 times

5 min 72°C

hold at 4°C

PCR products were visualised by electrophoresis on 1% agarose gels stained with ethidium bromide (0.01mg/ml). A 2 log base pair molecular weight marker (New England Biolabs, UK) was used to estimate the size of PCR products.

Purification of PCR product

Successful PCRs were repeated with a reaction volume of 60 µl. If a single, clear band on an agarose gel was obtained in the original PCR reaction, the PCR product was purified directly. Otherwise the PCR product was visualised by agarose gel electrophoresis and the appropriate gene fragment was cut out and purified. Direct purification or purification from agarose gel was performed using the High Pure PCR Product Purification Kit (Roche, UK).

Cloning

The cloning of successfully amplified gene fragments was carried out using the pGEM®-T Easy Vector System II (Promega, UK).

Preparation of agar plates

15 g agar was dissolved in 1000 ml LB medium. Immediately after the mixture had been autoclaved it was placed on a magnetic stirrer. Once the medium had cooled down to 50˚C, 0.1 g ampicillin was added and the mixture was divided between 20 plates. The plates were stored at 4˚C after having been allowed to solidify for approximately two hours. Before use, plates were air dried for around half an hour at RT.

Ligation

For the ligation reaction 5 µl 2X Rapid Ligation Buffer were mixed with 0.5 µl T4 DNA ligase and 0.5 µl pGEM®-T Easy vector. Deviating from the kit protocol, the 6 µl buffer, ligase, vector mix was divided in half, enabling two ligations to be performed. Two microliters of purified PCR product were added to the 3 µl mix and the ligation reaction took place overnight at 4˚C.

Transformation of *E. coli* cells

50 µl JM109 High Efficiency Competent E. Coli cells (Promega, UK) were originally aliquoted and stored at -80°C. For the transformation an aliquot was thawed on ice and 5 µl of ligation product was added. The mixture was incubated for 30-40 min on ice followed by a heat-shock at 42˚C for 30 sec. The cells were placed on ice for a further 2 min before 250 µl LB medium was added aseptically. The cells were shaken horizontally (225 rpm) at

37˚ for 1 h. In the meantime, 20 µl X-Gal and 100 µl IPTG were spread on an agar plate and dried for at least 15 min. Between 100 and 200 µl transformed cells were spread on the agar plate and the plates were incubated overnight at 37˚C.

Colony PCR

To confirm that selected colonies contained the correct insert, a colony PCR was performed. Before mixing the PCR reaction, an agar plate was prepared by dividing it into around 15 numbered fields. A white colony was picked using a sterile toothpick, dipped into the PCR mixture (containing forward and reverse primers within the cloned sequence) and streaked out onto a numbered field on the so called replica plate. The replica plate was stored at 37°C for a day and after that kept at 4°C. The PCR was performed as described above. The size of the PCR product was confirmed by gel electrophoresis, and the colonies containing the appropriate insert were picked from the replica plate and amplified in 3 ml LB medium and 3 µl ampicillin (50mg/ml) on a shaker overnight at 37°C. The following day 3 ml of the overnight culture were pelleted at 3000 rpm for 5 min. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, UK) according to the manufacturer's instructions.

Sequencing and analysis

Sequencing was carried out by MWG Operon (Germany) and The Genome Centre (Barts and the London School of Medicine and Dentistry, UK).

In vitro transcription

PCR amplification

To amplify DNA for in- vitro transcription 1 µl plasmid DNA was mixed with 10 µl Thermo pol Buffer (10 x), 10 µl dNTPs (2 mM), 5 µl Mr13 forward primer (10 mM;), 5 µl Mr13 reverse primer (10 mM), 68 µl H_2O and 1 µl Taq polymerase (5 u) to a total volume of 100 µl. The PCR was performed as described before with an annealing temperature of 50°C. After completion, the PCR product was purified using the High Pure PCR Product Purification Kit (Roche, UK) and dissolved in 30 µl elution buffer. The concentration was

measured by Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and 1 µl was run on an agarose gel.

Single-stranded RNA probes

The choice of the RNA polymerase depended on the orientation of the insert in the vector. Probes should be in a 3' to 5' (*i.e.* antisense) direction to enable them to bind to the endogenous mRNA in the cell. Depending on the orientation of the insert either the SP6 (Roche, UK) or T7 polymerase (Roche, UK) was used. One microgram of template, 2 µl DIG NTPs (10x ,DIG RNA Labeling Mix Roche, UK), 2 µl RNase-inhibitor (80 u, Roche, UK), 2 µl transcription buffer (10x, Roche, UK), 2 µl RNA polymerase (40 u, Roche, UK) were mixed, filled with Depc-treated H_2O to a volume of 20µl and left at 37°C for 2 h. To stop the reaction 2 µl EDTA (0.2 M, pH 8) was added. To precipitate the RNA-transcript 2.5 µl 4M LiCl and 75 µl pre-chilled 100% ethanol were added and left overnight at - 20°C. The next day the mix was centrifuged for 30 min at 13000 rpm at 4 °C. The pellet was washed in 70% ethanol and centrifuged 10 min at 13000 rpm and 4°C. After removing the ethanol and allowing the mix air dry for several minutes, it was dissolved in 50 µl DEPC-treated H_2O. The RNA probes were distributed into three tubes, two of which were stored at -80˚ and one at -20˚. Five microlitres of the resulting RNA probe were electrophoresed on an agarose gel to confirm the presence of a product of the anticipated size.

Double-stranded RNA

Double-stranded (ds) RNA was synthesised using the same protocol as for RNA probes with some modifications. Instead of DIG or fluorescein labelled NTPs, unlabelled NTPs (Roche, UK) were used. To obtain double stranded RNA the SP6 and T7 polymerases were added simultaneously. After precipitation, the dsRNA was diluted in 10 µl *Tribolium* injection buffer. To enable as many strands as possible to hybridise and form ds RNA, the 10 µl dilution was incubated for 2 min at 95˚C then placed into boiling water where it remained until it cooled to around 50˚C. The RNA was then taken out and kept at RT for another 10 min. Three microlitres were used to measure the concentration by Nanodrop spectrophotometer and verify the product size by gel electrophoresis. The dsRNA was stored at -80˚C or -20˚C if to be used during the next 2 months.

RNA-interference (RNAi)

Embryonic RNAi

Collection, dechorionisation and preparation

Beetles were transferred to white flour and kept for one and a half hours at 32˚C before egg collection. Eggs were subsequently kept for a further half an hour at 32˚C, following collection. To dechorionate embryos, they were washed in tap water, followed by 40 seconds in chlorine bleach diluted 1:1 in tap water. They were then washed for 15 seconds in tap water followed by a further 30 second incubation in bleach solution. The dechorionated embryos were then washed a further three times in tap water (15 seconds per wash). Embryos were transferred with a broken glass pipette onto a slide. Using a single eyelash glued to a glass capillary, embryos were arranged in a row in the middle of a slide leaving a gap between individual embryos. Approximately 80 to 100 embryos, all facing the same direction, were aligned on each slide. After the embryos were dried for 10 minutes, they were immersed in Voltalef oil. Slides with mounted embryos were placed in a petri dish and stored at 32˚C until injection.

Injection

Embryos were injected under a 40x objective of a compound microscope. Approximately 1 µl of dsRNA (concentration varying between 1.5 and 3.5 µg/µl) was loaded into a glass capillary (OD =1 mm; ID = 0,58 mm; length = 10 cm). The glass capillaries had been pulled with a Kopf Instrument (heat: 11.5, solenoid 2.2) and bevelled on a micro grinder for 2 min. Embryos were injected at the anterior end.

Fixation of RNAi embryos

Once the embryos had reached the appropriate stage, they were separated from the slide using a single eyelash. Via a small spatula, they were transferred into the fixation mix. The fixation procedure was the same as for wild type embryos.

Pupal and adult injection

Pupal and adult injections were performed as described in Posnien *et al.* (2009).

Morphological staging

The development of the nervous system occurs during a given developmental time period in *Tribolium* embryogenesis. To be able to understand and use descriptions of early neurogenesis in *Tribolium*, it is necessary to correlate them to specific morphological stages in embryogenesis. Unfortunately, there is currently no published staging available for *Tribolium castaneum* embryogenesis as there is for the fruit fly *Drosophila melanogaster* or the cobweb spider *Parasteatoda tepidariorum*, for example (Mittmann and Wolff 2012; Campos-Ortega and Hartenstein 1985). Handel *et al.* (2000) describe the development from blastoderm until serosal closure in great detail, with an emphasis on cell shape and movement, but do not establish a morphological staging system. It is common practice to give the hours of development after egg laying (AEL) at a chosen temperature, typically at 30˚C or 32˚C. However, even under conditions of identical temperature and humidity embryos can vary in their pace of development (Handel *et al.* 2000; personal observation). Furthermore, embryos treated with double stranded RNA (RNAi embryos) exhibit a delay in development. Thus, it is necessary to provide a staging system based on morphological landmarks, to enable reliable interpretation of especially data of functional studies. The staging presented in this thesis was primarily constructed as a tool for aiding interpretation of neurogenesis, and therefore only includes embryonic stages relevant to this purpose. Nuclei stainings of *Tribolium* embryos were analysed under a fluorescence microscope to establish the staging system. The landmarks used were the number of segments, the developmental stage of appendages, the length of the embryo along the anterio-posterior (AP) axis and the width along the dorsal-ventral (DV) axis proportionally to the egg size. Eggs were fixed from 9 h till 48 h AEL every hour. The eggs were allowed to develop at 32°C. From 13 to 23 h, embryos were staged every hour. Thereafter only the following four stages are described in detail 25 - 26 hours; 28 - 29 hours; 38 - 40 hours and 44 - 46 hours. In total eight embryos per stage were analysed to establish the staging system.

Development of *Tribolium*

The development from the zygote to the fully developed embryo lasts around 69 hours at 32°C in *Tribolium* (Bucher 2009). The starting point of neurogenesis, and therefore the starting point of the staging presented here, is defined as the onset of the expression of the proneural gene *Tc-ASH*, at around nine hours AEL at 32˚C. This work, however, focuses on mechanisms of neurogenesis occurring mainly between 13 and 48 hours AEL at 32˚C. Hence, only stages in this timeframe, with the exception of the first stage (9 AEL), are analysed in detail. For the purpose of this thesis the development of the nervous system was divided into 15 stages, termed NS1 to NS15. The segments of *Tribolium* form consecutively, which is characteristic for short germ insects (see review Liu and Kaufman 2005). The first ten developmental stages are characterised by the formation of segments, resulting in an elongation of the germ band. Once all ten segments have developed the germ band retracts along the AP axis but broadens along the DV axis. In the following the 15 stages are described in detail with the respective example stages depicted in Figure 2-1.

Stage NS1

During NS1 the germ band comprises the head lobes, the gnathal region which will develop into the future mandibles, maxillae and labium, and the growth zone. Segments are not yet distinguishable.

Stage NS2

The gnathal segments have broadened along the AP axis and the thoracic rudiment is formed. Gnathal segments begin to be distinguishable as contractions in edge of the germ band cause indentation along the AP axis. Thoracic segments can not be distinguished yet. The germband, except for the growth zone at the posterior end of the embryo, is similar in width.

Stage NS3

All three thoracic segments and the first abdominal segment are demarcated (in the anterio-posterior axis) by intersegmental furrows (arrow).

Stage NS4

Stage NS4 is characterised by a lengthening of the existing segments and a width change along the DV axis, with the first thoracic segment being the widest. The first three abdominal segments are added and increase in width as the embryo develops. In this stage the segments are properly distinguishable by the intersegmental furrow (arrows).

Stage NS5

During stage NS5 four additional abdominal segments are formed and the growth zone lengthens.

Stage NS6

During the next stage (NS6) appendages start to form, and are initially visible as buds to either side of the midline. Furthermore, the antennae are visible and seven abdominal segments have formed.

Stage NS7

The appendages gain in size with the maxillae exhibiting a triangular shape and the labrum appears as a paired lobe anterior to the stomodeum. Additionally, eight abdominal segments have been formed by stage NS7.

Stage NS8

All 10 abdominal segments and the proctodeum have formed with the posterior ones still increasing in size. The thoracic legs have further lengthened now partly covering the posterior hemisegments (segment borders indicated by dashed line). Between stage NS8 and NS9 the embryo has reached its greatest length.

Stage NS9

This stage is characterised by further lengthening of the legs and differentiation of the maxillae. The pleuropodia, appendages on the first abdominal segment emerge. The abdominal segments now all have a similar width. From this stage onwards segments start to condense along the AP axis and simultaneously gain width along the DV axis, resulting in a retraction of the embryo along the AP axis.

Stage NS10

The legs gain further length growing medially towards each other. Additionally, the antenna and labrum differentiate further.

Stage NS11

The legs reach over the midline occasionally touching each other. The gnathal appendages differentiate further and still gain length. The distal tip of the pleuropodia differentiates into a small nodule (arrow).

Hereinafter, the stages are not described hourly. Stages are selected every three to four hours of development.

Stage NS12

This stage is characterised by the invagination of the proctodeum, observable at the posterior tip of the germ band. The segments have noticeably condensed along the AP axis and broadened along the DV axis. Between stage NS11 and NS12 gnathal segments begin to condense.

Stage NS13

The hindgut extends anteriorly over the last abdominal segment. The legs reach over the subsequent segment. Tracheal pits are visible in all thoracic segments and the first two to three abdominal segments (arrows).

Stage NS14

The hindgut reaches anteriorly the eighth abdominal segment. The tips of the legs are extending into the anterior part of the second segment away.

Stage NS15

In the last stage analysed at around 48h AEL at 32˚C, the embryo exhibits an oval shapez and its lenght is almost half the size of the germ band at its longest elongation (between stage NS8 and NS9). The segments have roughly halfed their width and the gnathal segments are fused with the head lobes forming the head. In addition, the hindgut developed into a tubelike structure. The legs extend posteriorly to cover three posterior segments.

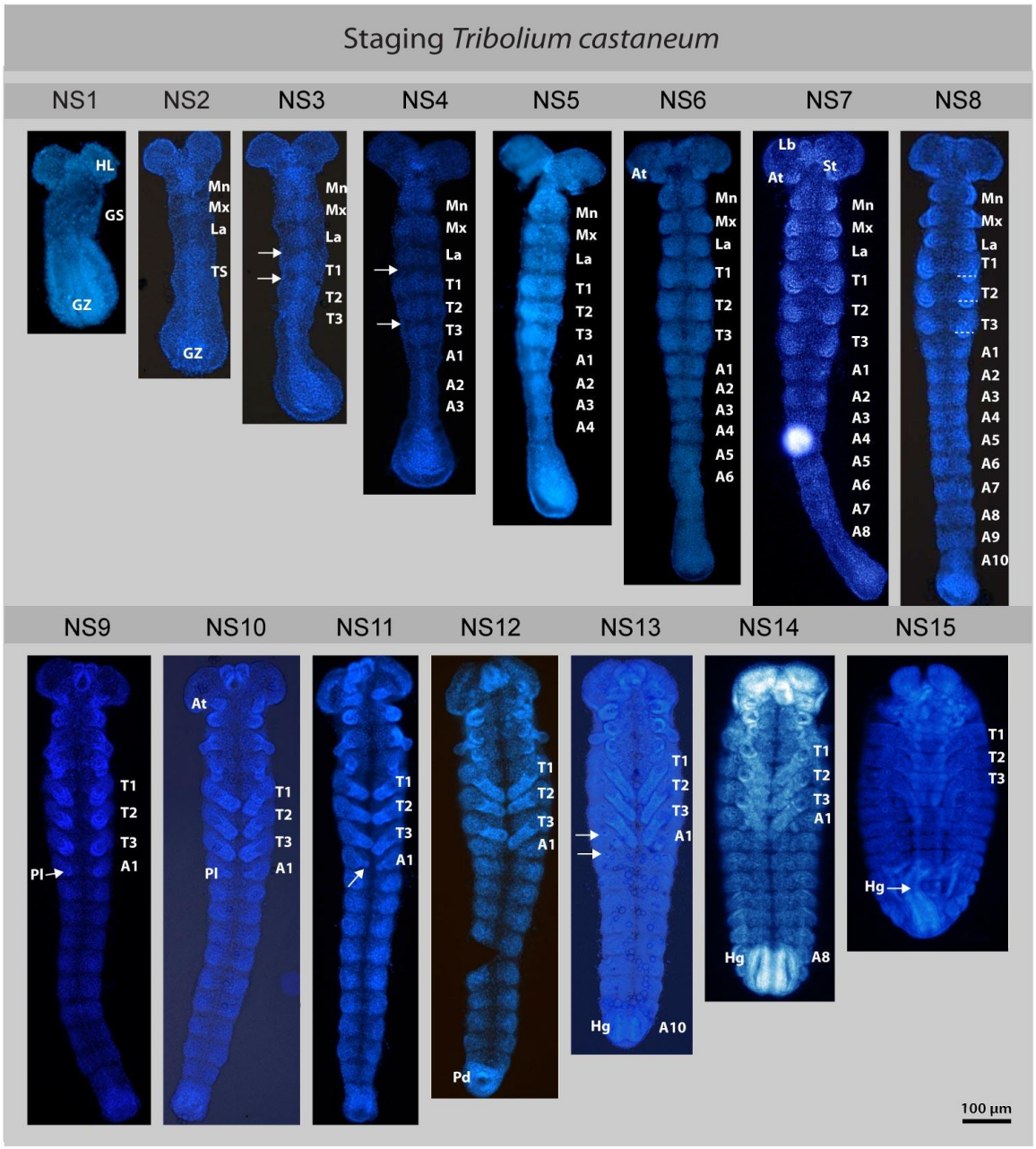

Figure 2-1: Morphological staging of embryological development in *Tribolium castaneum* Embryos are stained with the nuclei marker Hoechst. The development is divided into 15 embryonic stages relevant for neurogenesis. NS2 to NS11 are staged in hourly intervals. NS12, NS13, NS14 and NS15 are staged 25-26 h, 28-29 h, 38-40 and 44-46 h AEL, respectively. Embryos were developed at 32˚C. Anterior is towards the top. (NS3 and NS4) Arrows point towards the intersegmental furrow. (NS11) Arrow points towards nodule at the tip of the pleuropodia. (NS13) Arrows point towards tracheal pits. Antenna (At); rudiment of gnathal segments (GS); growth zone (GZ); hind gut (Hg); head lobes (HL); pleuropodia (Pl); labrum (La); labium (Lb); mandible (Mn); maxilla (Mx);rudiment of thoracic segments (TS) first thoracic segment (T1); second thoracic segment (T2); third thoracic segment (T3); abdominal segments 1-10 (A1-A10); proctodeum (Pd); scale bar = 100 µm.

Documentation and analysis

Prior to analysing the embryos under the microscope, flat preparations were made. The embryos were placed on a slide in a drop of glycerol and dissected using a stereomicroscope. Using tungsten needles the yolk was carefully removed. In some cases, the legs had to be removed to enable a clear picture of the nervous system.

Microscopy

Colorimetric stainings were analysed using a Leica DM IL HC microscope. For fluorescent *in situ* hybridisation, antibody staining and phalloidin staining a Leica SP5 confocal microscope was used.

Analyses

Confocal microscope image stacks were analysed using the 3D reconstruction software Imaris (Bitplane AG, Switzerland). The extended-section mode enables the visualization of horizontal, transverse and sagittal sections of variable density, depending on the number of pictures superimposed. The surpass mode (volume mode) constructs a 3D picture and allows the combined visualisation of cells in different positions along the apico-basal axis. In combination with the ortho-slicer tool, which enables the visualization of single sections, spots can be manually added. A combination of the described modes and tools was used to analyse the neuroblast pattern (chapter 3.1), the gene expression pattern of specific neuroblasts (chapter 3.1 and 3.2) and the expression of Even-skipped and Tailup in neurons (chapter 3.3).

Photoshop and Helicon Focus (Helicon Soft Ltd., Ukraine) were utilised to edit and assemble pictures of fluorescent and colorimetric stainings. Picture plates were composed in Adobe Illustrator.

3 Results

The following results chapter is divided into three sections – 'Morphological overview of early neurogenesis and formation of neuroblast pattern', 'Columnar gene expression in specific neuroblasts of *Tribolium*' and 'Functional studies of columnar genes and their influence on neural subtype specific gene expression'. The first part provides an overview of the morphological development of the nervous system in *Tribolium*, followed by the establishment of a neuroblast map and gene expression pattern of neuroblasts. Subsequently, the expression patterns of the columnar genes *Tc-vnd, Tc-ind* and *Tc-msh* in the ventral nervous system and specific neuroblasts are analysed, using the neuroblast map previously established. Finally, the impact of columnar genes on neural identity of neuroblasts is described by examining the expression pattern of the neural subtype specific genes *even-skipped* and *tailup* in wild type embryos and *Tc-vnd*^{RNAi} phenotypes.

3.1 Morphological overview of early neurogenesis and formation of neuroblast pattern

Morphological overview of neurogenesis

Morphological development of the nervous system in the thoracic segments was analysed using a combination of nuclei and cell membrane markers. Nuclei were stained with either Hoechst or SYBR® Green. The latter was chosen to visualise nuclei in later stages, where several layers of cells may be present, as it provided better resolution than Hoechst. The cell shape was visualised using either an antibody against alpha-tubulin or the phallotoxin Phalloidin which binds to F-actin. To visualise the axonal pattern in later stages an antibody against horseradish peroxidase (HRP) was used. Jan and Jan (1982), demonstrated that membrane glycoproteins selectively recognised by anti-HRP antibodies are expressed on the surface of neural tissue in *Drosophila* and a grasshopper species. Data presented here demonstrate that it can also be used as a neuronal marker

in *Tribolium* (see also Ewan and Bashaw 2012). Confocal microscopy and the use of 3D image analysis software enabled the investigation of morphological developments along the horizontal, sagittal and transverse axes of *Tribolium* embryos.

The germband of stage NS3 *Tribolium* embryos consists of one apical layer of ectodermal cells and several layers of mesodermal cells basal to them (Fig. 3-1.1 A'). The ectodermal cells are rectangular (Fig. 3-1.1 A'). Mesodermal cells are rounder and smaller than ectodermal cells (Fig. 3-1.1 A', B', C'). At the onset of neurogenesis the two cell types are adjacent to each other (Fig. 3-1.1 A'). Subsequently some ectodermal cells from the neuroectoderm develop into neuroblasts and delaminate to form a distinct layer between ectodermal and mesodermal cells (Fig. 3-1.1 C', D'). Concurrently, mesodermal cells move towards the lateral part of the segment. Early neuroblasts can be easily distinguished by their appearance. A fully delaminated neuroblast is a round cell exhibiting a large nucleus (Fig. 3-1.1 B, C, D). Ectodermal cells which adopt the fate of neuroblasts change their shape during delamination, becoming longer with the nuclei at the basal end and the cell membrane still attached to the ectoderm (Fig. 3-1.1 C'). Eventually they form triangular shaped cells, prior to the cell membrane detaching completely from the ectoderm (Fig. 3-1.1 D'). Comparison of embryos at similar stages demonstrates that the time at which thoracic neuroblasts in similar positions delaminate varies significantly. Nevertheless, it appears that a certain number of specific neuroblasts delaminate in a given developmental period. The first neuroblasts delaminate between stage NS3 and NS4. They are arranged along the medial and lateral column of a hemineuromere (Fig. 3-1.1 B, B'). Around stage NS5 three columns of neuroblasts have formed per hemineuromere (Fig. 3-1.1 C). From stage NS6 to NS7 six to seven rows of neuroblasts consisting of three to four columns of neuroblasts respectively are detected (Fig. 3-1.1 D). Neuroblast numbers increase until around stage NS11. A maximum of 30 neuroblasts plus the median neuroblast (MNB) were detected in any single hemineuromere. By stage NS12 the number of recognizable neuroblasts has already decreased. The remaining neuroblasts which can be distinguished in NS13 lie most apically in the former ectoderm layer (Fig. 3-1.1 E, E'). In the oldest embryos analysed (stage NS15), it was not possible to distinguish neuroblasts from other surrounding cells by morphology. Whilst the neuroblast pattern is formed, the embryo develops further and segments condense along the AP axis but gain width along the DV axis. This influences

the movement of neuroblasts. Simultaneous with the formation and delamination of neuroblasts, previously delaminated cells divide and bud-off GMCs basally inside the embryo (Fig 3-1.1 D'), which then divide into neurons or glial cells. The division of GMCs into neurons and glial cells was not analysed in detail in the present work, however, and this assumption is based primarily on the increase in cell numbers along the apico-basal axis (Fig. 3-1.1 E').

Early axons are formed from stage NS9 onwards (data not shown). At stage NS13 the anterior commissure has formed and the neuropil is highly developed but the connectives are only starting to meet at the adjacent segments (Fig. 3-1.1 F). In the latest stage analysed (NS15) the nervous system exhibits the rope ladder-like formation characteristic of arthropods (Fig. 3-1.1 H, G, G'). The axonal scaffold is positioned between a layer of 6 to 8 cells basally and one layer of cells apically to it (Fig. 3-1.1 G').

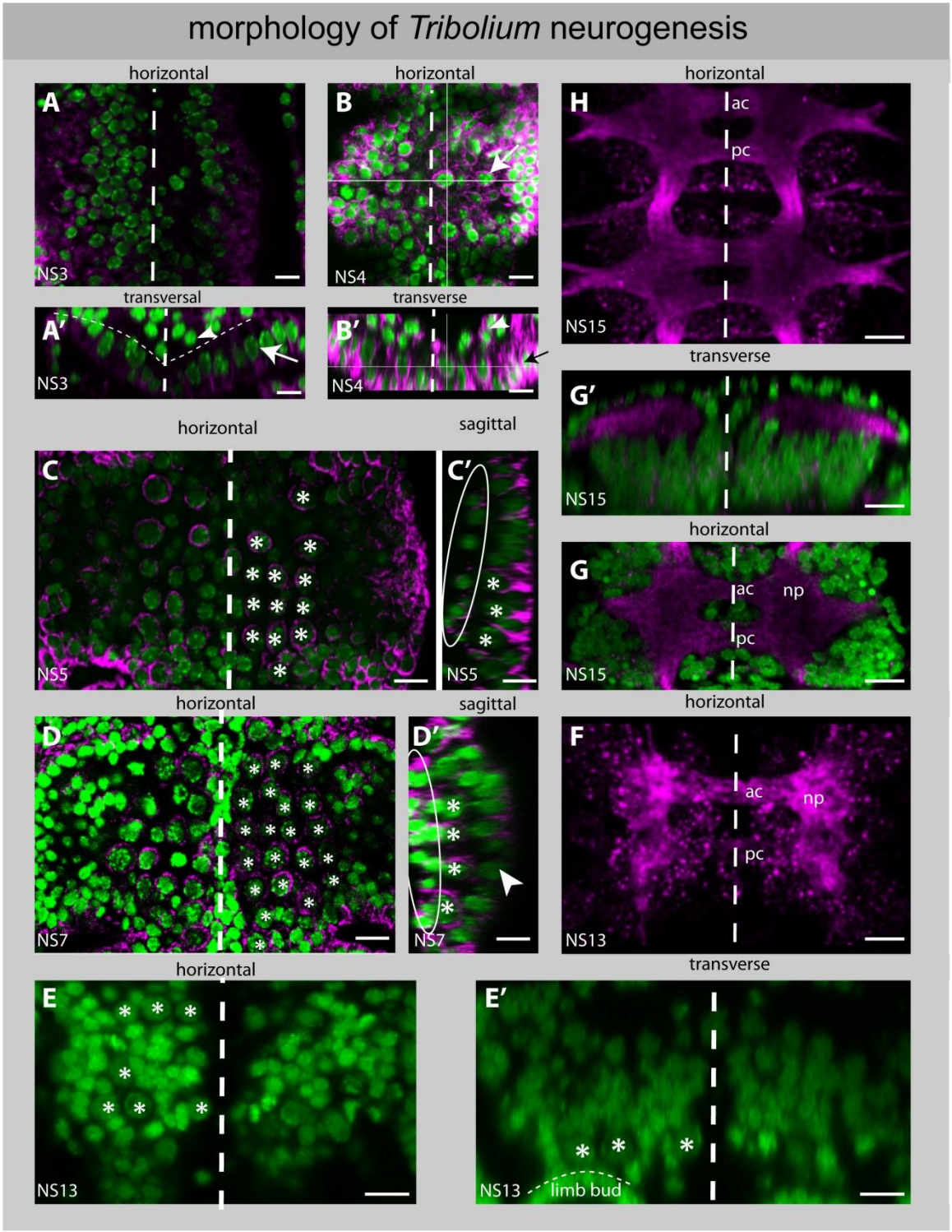

Figure 3-1.1: Morphology of *Tribolium* neurogenesis.

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Neuroblast arrangement in the germ band of *Tribolium* embryos. Confocal micrographs of flat preparations of the first thoracic segment. Hoechst nuclei stain (green) (A-D), Sytox (green) (E, E', F, G, G'); α-tubulin labeling of cell membranes (magenta) (A-D'); HRP antibody labeling of axonal scaffold (magenta) (F, G, G', H). Anterior is towards the top. (A, A', B, B') Horizontal section with the respective transverse section. Basal is towards the top (A', B'). (A) Apical layer of ectodermal cells in a stage NS3 embyro. (A') Ectodermal cells (arrow) are positioned apically with a layer of mesodermal cells basal to them. Note brighter green in mesodermal cells (arrowhead). (B) The first neuroblasts are formed in the medial (white cross) and lateral (arrow) column. (B') Neuroblasts are positioned in a layer between ectodermal (arrow) and mesodermal cells (arrowhead). (C, C', D, D') Horizontal sections with the respective sagittal sections. Basal is to the left (C', D'). (C) Focus on the neuroblast layer with three columns of neuroblasts (asterisks). (C') Several neuroblasts are in the process of delaminating with the cell membrane still attached to the ectoderm (asterisks). Basally to the neuroblasts a layer of mesodermal cells is visible (white oblong). (D) At stage NS7 four columns of neuroblasts have formed (asterisk). (D') Three distinct layers of apically ectodermal cells (arrowhead), medially fully delaminated neuroblasts (asterisks) and ganglion mother cells (white circle) have formed. Note the brighter green of ganglion mother cells. (E) Horizontal section. At stage NS13 numbers of recognizable neuroblasts have increased. Note that there are more neuroblasts than visible in the section presented, due to curvature of neuromere. (E') Transverse section of E. Neuroblasts are positioned apically (asterisks), with several layers of GMCs and neurons basally to them. (F) Embryo at the same stage as that shown in E (NS13). Focus on the axonal scaffold. The anterior commissure is highly formed, whereas the posterior commissure is barely visible. Additionally, the connectives are not yet fully developed. (G) Horizontal section of embryo at stage NS15. The axonal scaffold appears to be fully developed. (G') Transverse section of G. The axonal scaffold is positioned basally with several layers of neurons apically and a layer of one to two neurons basal to it. (H) Horizontal section of embryo at stage NS15 with focus on the characteristic axonal scaffold. The midline is indicated by a vertical dashed line; anterior commissure (ac); posterior commissure (pc); neuropile (np); scale bar = 10 µm.

Neuroblast formation visualised by the pan-neural genes *Tc-asense* and *Tc-prospero*

Before neuroblast delamination in the thoracic segments is analysed in detail an overview of neuroblast formation in the whole embyro is provided, using the expression pattern of the neuroblast specific gene *Tc-ase* (Wheeler *et al.* 2003). *Tc-ase* expression is first detected between stage NS3 and NS4 in two neuroblasts in the middle of the first thoracic segment and the growth zone (Fig. 3-1.2 A). Subsequently, *Tc-ase* expression extends to the maxillary and labial segments and the second and third thoracic segment (Fig. 3-1.2 B), followed by expression in the brain (Fig. 3-1.2 C). During stage NS4 and NS5 neuroblasts are continously formed in the brain, maxiallary, labial and thoracic segments (Fig. 3-1.2 D). Eventually at stage NS7 *Tc-ase* expression is detected in the mandibular and intercalary segments (Fig. 3-1.2 E). Furthermore, *Tc-ase* is expressed in the stomodeum (Fig. 3-1.2 E). Abdominal segments gradually start to express *Tc-ase* when around 15 neuroblasts are present in the thoracic hemisegments (Fig. 3-1.2 F). During the next two stages neuroblast formation in thoracic and abdominal segments continues (Fig. 3-1.2 G, H) until eventually all neuroblasts in all segments have formed around stage NS12, as revealed by a balanced expression of *Tc-ase* in all segments (Fig. 3-1.2 I). Henceforth, *Tc-ase* expression decreases (Fig. 3-1.2 J, K), until no *Tc-ase* expression in any of the neuromeres is detectable at the last stage analysed (NS15) (Fig. 3-1.2 L). The strong expression remaining visible in the pedipalps is background staining (Fig. 3-1.2 K, L).

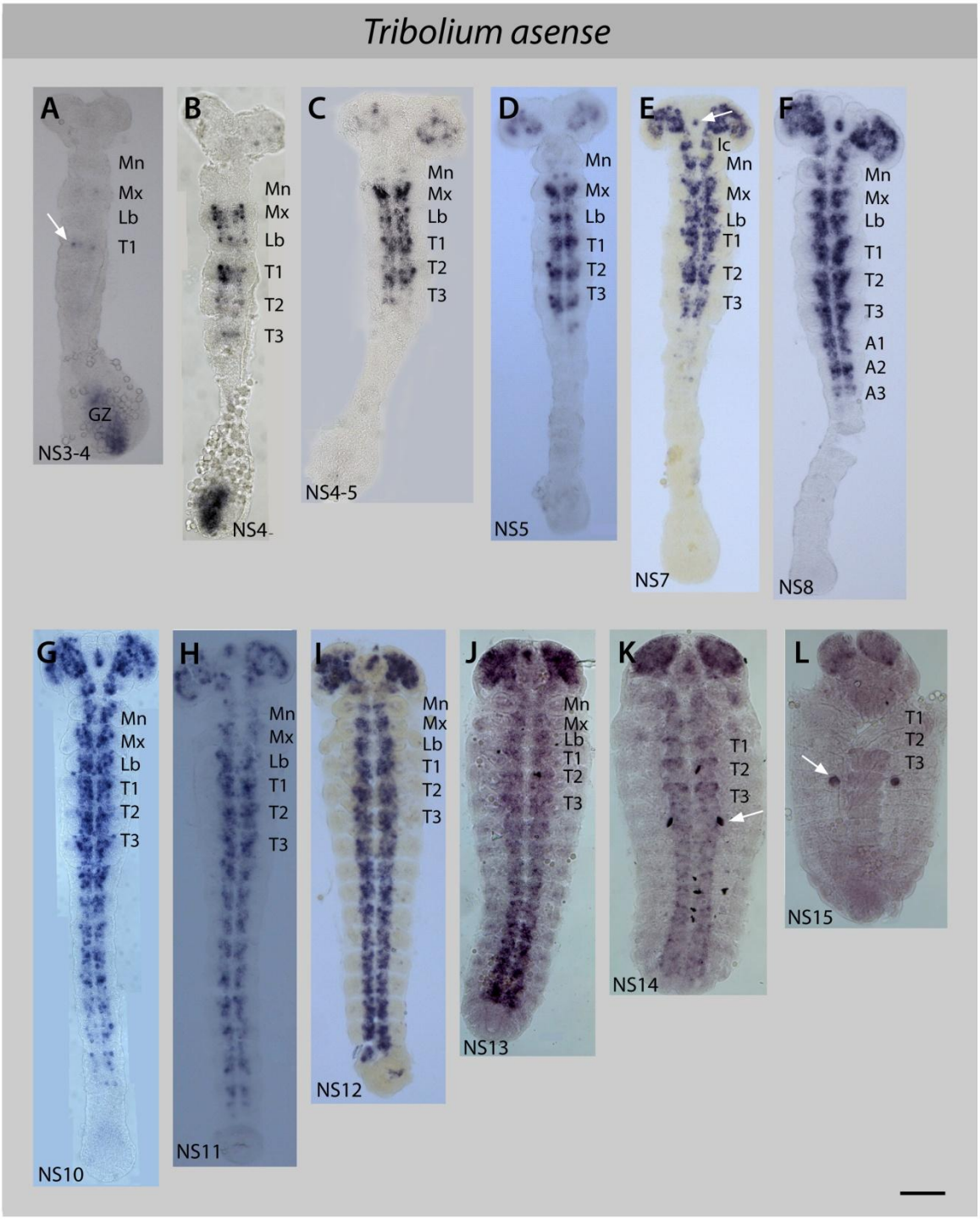


Figure 3-1.2: Neuroblast formation in consecutive developmental stages of *Tribolium*

Figure 3-1.2: Neuroblast formation in consecutive developmental stages of *Tribolium*

Neuroblasts are visualised by *Tc-ase* expression detected by *in situ* hybridisation (A-L). Consecutive developmental stages are shown (stages NS6 and NS9 missing). Anterior is towards the top. (A) *Tc-ase* expression is first detecteable between stage NS3 and 4 in a single neuroblast in the first thoracic segment (arrow). Additionally expression in the growth zone (GZ) is detected. (B) More neuroblasts are formed over the next stage and the expression of *Tc-ase* expands anteriorly into the labial and maxillary segments and posteriorly into thoracic segments T2 and T3. (C, D) Stage NS4 to 5 is characterised by an increase in the number of neuroblasts in the already *Tc-ase* expressing segments, the formation of neuroblasts in the brain and a cessation of *Tc-ase* expression in the growth zone. (E) At stage NS7 numbers of neuroblast further increase and neuroblasts in the mandibular and intercalary segment are formed. Additionally *Tc-ase* expression in the stomodeum is detected (arrow). (F-H) In the next three stages abdominal segments gradually start forming neuroblasts and the number of neuroblasts in the remaining segments increases. (I, J) From stage NS12 onwards a decrease of *Tc-ase* expression in the thoracic segments is visible. (K) At stage NS14 expression of *Tc-ase* is restricted to the anterior part of most segments and the brain. (L) At NS15 *Tc-ase* expression is no longer detectable in the ventral nervous system. (K, L) Note strong background staining in pleuropodia (arrow). For abbreviations see abbreviation index page 10; scale bar = 100 µm.

The pan-neural gene *prospero* is essential for specifying neuronal fate of neuroblasts in *Drosophila* (Doe *et al.* 1991). In this study it was used as a marker to analyse early neuroblast formation (Fig. 3-1.3). *Tc-prospero* (*Tc-pros*) is first detected between stage NS1 and NS2 in two ectodermal cell clusters per segment and the head (Fig. 3-1.3 A). Out of the *Tc-pros*$^{+}$ cell cluster arise the first *Tc-pros*$^{+}$ midline precursor cells 2 (MP2). Shortly after, expression is observed in the midline precursor cell 1 (MP1) (Fig. 3-1.3 B, B'; Fig.3-1.5 B). Subsequently all neuroblasts appear to express *Tc-pros*. Initially the expression is very faint (Fig. 3-1.3 B, B'; Fig. 3-1.6 A) but eventually becomes stronger (Fig. 3-1.3 C, D, D'). Around stage NS10 strong expression in the MNB is detectable (Fig. 3-1.3 C). Furthermore, *Tc-pros* is detected in some GMCs (Fig. 3-1.3 F). Except for the early expression at stage NS2 (Fig. 3-1.3 A) no *Tc-pros* expression was detected in ectodermal cells (Fig. 3-1.3 E') and neurons. As *prospero* mRNA and protein are asymmetrically distributed in the cytoplasm during ganglion mother cell formation in *Drosophila* (Broadus *et al.* 1998, Li *et al.* 1997, Spana and Doe 1995) this was investigated in *Tribolium*. In non-mitotic *Tribolium* neuroblasts *Tc-pros* mRNA is distributed evenly in the cytoplasm (Fig. 3-1.3 F). At the onset of mitosis *Tc-pros* mRNA assembles apically to the nucleus (Fig. 3-1.3 E, E'). In *Drosophila pros* transcript subsequently moves basally and is passed on to the GMC. In *Tribolium* no neuroblast with basal *Tc-pros* transcript during mitosis was observed. However, neuroblasts negative

for *Tc-pros* mRNA with a GMC basal to them with apical *Tc-pros* mRNA in the GMCs were detected (Fig. 3.1-3 F).

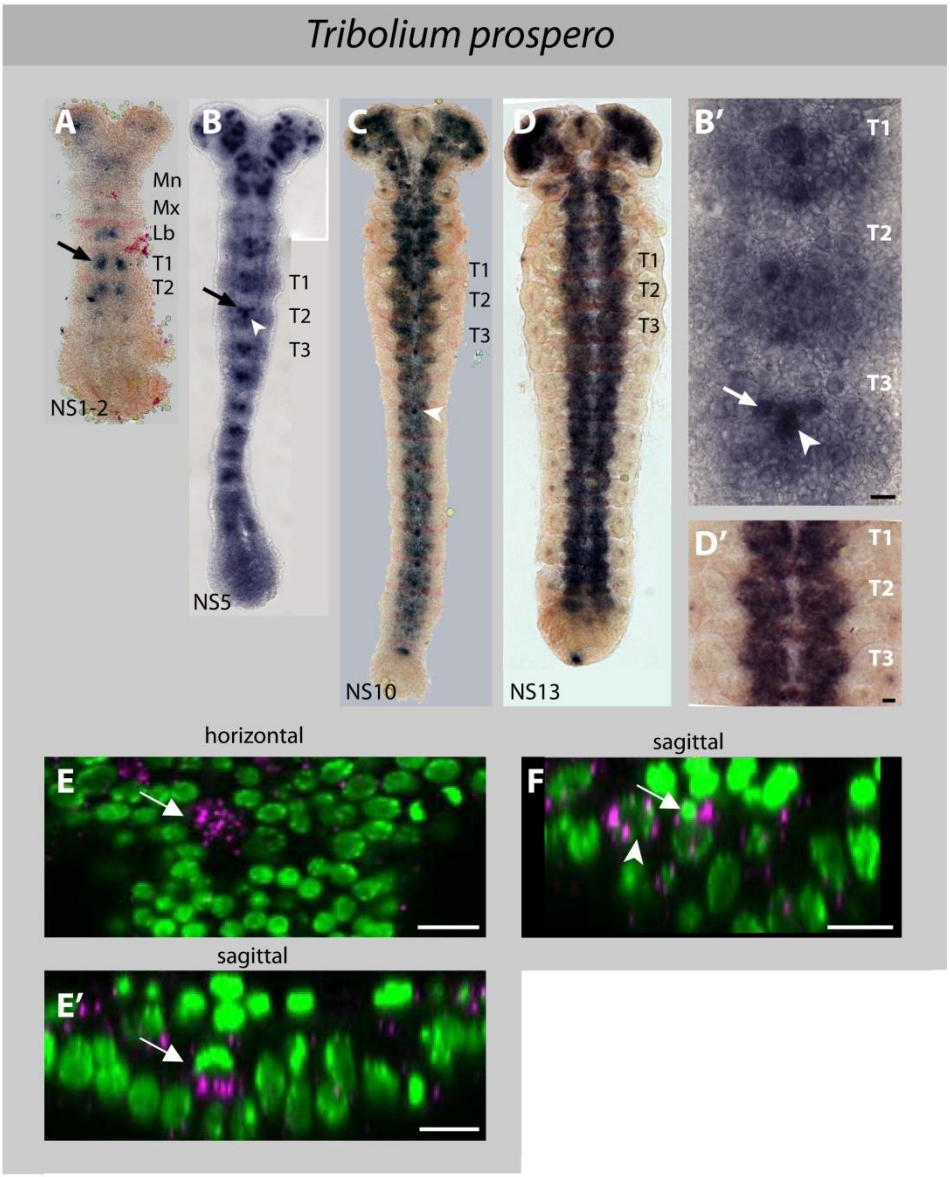


Figure 3-1.3: *prospero* expression in *Tribolium*

Tc-pros RNA detected by chromogenic (A-D') and fluorescent (E-F, magenta) *in situ* hybridisation. Additionally, in some embryos (A, C, D, D') Engrailed expression is detected by antibody staining (red). Hoechst nuclei stain (green) (E, F). Anterior is towards the top. (A) *Tc-pros* expression is initially detected in clusters of ectodermal cells in the middle of a hemisegment (arrow) in stage NS1-2 embryos. (B, B') First expression in neural cells is detected in midline precursor cell 2 (MP2, arrows) and midline precursor cell 1 (MP1, arrowhead). Note faint expression in neuroblasts (B'). (C, D, D') Expression in neuroblasts intensifies and expands into all neuroblasts and some GMCs. Note strong expression in MNB in C (arrowhead). (E, E') Horizontal and sagittal section (basal towards the top) illustrating *Tc-pros* expression apical to a neuroblast nucleus (arrow). (F) *Tc-pros* is distributed evenly in a non-mitotic neuroblast (arrowhead). Furthermore, it is also detected in GMC with no expression in neuroblast apical to it (arrow). For abbreviations see abbreviation index page 10; scale bar = 10 µm.

Establishment of neuroblast pattern

The preceeding description of neurogenesis and the expression pattern of *Tc-ase* served to provide an overview of early neurogenesis at the scale of the whole embryo. Henceforth, a detailed analysis of the formation of the neuroblast pattern in the three thoracic segments follows, eventually resulting in a neuroblast map. Neuroblast development was primarily analysed using the nuclear marker Hoechst and Sytox and α-tubulin staining. Around 15 embryos of each stage between NS4 and NS12 were analysed to establish the neuroblast map. In addition *Tc-pros* expression was used to aid the identifiaction of specific early formed neuroblasts. Neuroblasts were named according to the conventions applied in existing neuroblast maps for *Drosophila* and *Schistocerca* (Doe 1992, Broadus *et al.* 1995, Bate 1976). Neuroblasts are identified by two numbers, with the first one defining the position along the anterio-posterior axis in a segment (rows) and the second number defining the position along the medio-lateral axis (columns). As in other species of insects analysed, *Tribolium* has seven rows of neuroblasts with varying numbers of columns. In addition to presenting the final map (Fig. 3-1.4), -characteristics which distinguish specific neuroblasts are described (Fig. 3-1.5).

Data presented concerning *Tc- ase* expression (Fig. 3-1.3) has illustrated that there is an early gradient in neuroblast formation between the three thoracic segments. In later stages, however, the difference between the three segments becomes less pronounced. To obtain the neuroblast map all three thoracic segments were analysed. In *Drosophila* the formation of the neuroblast pattern in a single thoracic segment was originally divided into three, and then later into five, segregation waves (early S1, S1 to S5) (Hartenstein and Campos-Ortega 1984, Doe 1992). In *Tribolium* neuroblasts form sequentially. Nevertheless at different stages a certain number of neuroblasts in similar position is detected and therefore neuroblast development was divided into four different stages – NS4, NS6, NS8 and NS11- using the developmental stages described before. By stage NS4, five to six neuroblasts have fully delaminated (Fig. 3-1.4 A –A'''). NB 5-2 and the midline precursor cell MP2 can be assigned to specific neuroblast on the basis of their position. In addition the midline precursor MP2 is the first neural precursor cell to express *Tc-pros* (Fig. 3-1.5 A). The MP2 is furthermore characterised by its elongated appeareance (Fig. 3-1.5 B) and in embryos at stage NS6 it is the most basally positioned

neural precursor cell. Between stage NS4 and NS6 three columns with seven rows of neuroblasts respectively are formed. In addition to MP2 which is formed in both hemisegments, one midline precursor cell (MP1) is formed along the midline (Fig. 3-1.4 B –B’’’, Fig. 3-1.5 B. B’). Using the midline precursor cells and the morphological segment boarders as indicators, the neuroblasts of the first column can be assigned to NBs 1-1, 2-2, 4-1, 5-2, 6-2 and NB 7-1. In the middle column NBs 1-2, 3-2, 4-2, 5-3, 6-3 and 7-2 can be assigned to specific neuroblasts using their position and the position of subsequently forming neuroblasts as cues. Only neuroblasts 6-4 and 7-4 can be assigned to specific neuroblasts in the lateral column. Between stage NS6 and NS8 lateral neuroblasts in the anterior part of the hemineuromere delaminate, resulting in four columns in row two, three, four and five (Fig. 3-1.4 C-C’’’). Additionally, NB 3-1 forms medially to NB 3-2, and NB 6-1 forms between NBs 6-2 and 7-1 (Fig. 3-1.4 C’’’, Fig. 3-1.5 C). At stage NS8 the MP2 has divided into two smaller cells in most embryos or is in mitosis (Fig. 3-1.5 B). Furthermore, the median neuroblasts (MNB) is formed in the posterior part of the segment. Two more neuroblasts are formed in row five laterally to neuroblasts 5-2 and 5-3 and can now be identified as NBs 5-4 and NB 5-6. At stage NS11 the final neuroblast array is formed with three row one neuroblasts, five row two, three and five neuroblasts and four neuroblasts in row four, six and seven, respectively (Fig. 3-1.4 D-D’’’). The midline precursor MP1 divides shortly after stage NS8. Additionally NB 5-1 delaminates next to the midline in row five (Fig. 3-1.5 C).

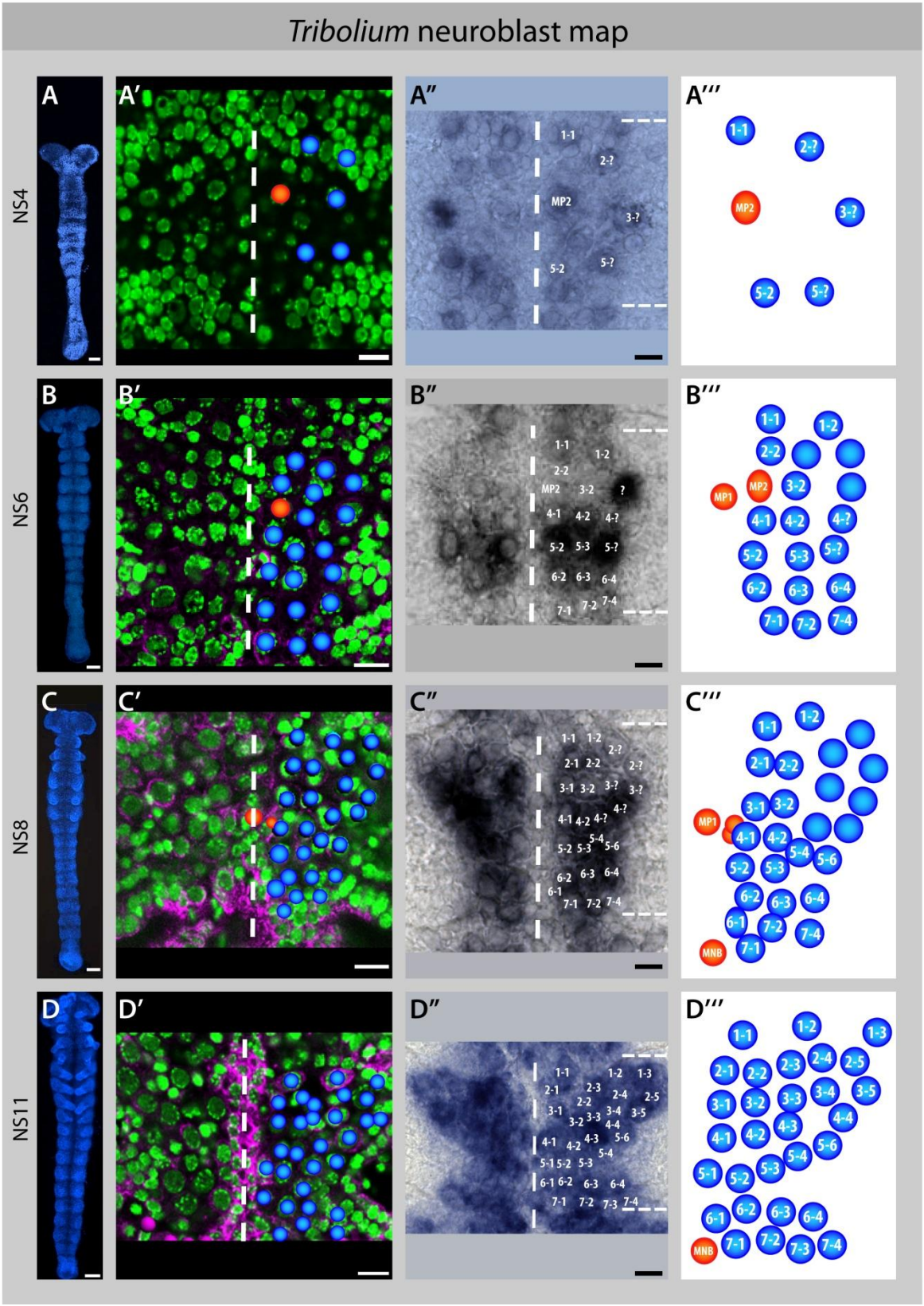


Figure. 3-1.4: *Tribolium* neuroblast map at four different developmental stages

Figure. 3-1.4: *Tribolium* neuroblast map at four different developmental stages

Embryos are stained with the nuclei marker Hoechst (green) and cell membranes are labeled with α-tubulin (magenta) (B', C', D'). Anterior is towards the top. (A-D) Whole embryos at stages NS4, NS6, NS8 and NS11. (A'-B') Horizontal section of T1 segment with neuroblasts depicted in the right hemineuromere. (A''-B'') T1 neuromere with neuroblasts detected by *in situ* hybridisation against *Tc-ase* mRNA. (A'''-D''') Schematic neuroblast map (neuroblasts are in blue, MP1, MP2 and MNB are in orange). (A-A''') At stage NS4 five neuroblasts of row 2, 3 and 5 are detectable in the neuroblast layer. NBs 5-2 and 1-1 can be identified. The other neuroblasts can not be assigned to specific identities. Additionally MP2 is formed. (B-B''') At stage NS6 three columns and seven rows of neuroblasts have formed. All neuroblasts can be assigned to specific neuroblasts identities except for the most lateral neuroblasts in row two, three, four and five and the intermediate neuroblast in row two. (C–C''') At stage NS8 more lateral neuroblasts are formed. Additionally NB 6-1 forms directly beside the midline between NBs 6-2 and 7-1 and the median neuroblasts (MNB) is detected in the posterior part of the segment. Note that MP2 has divided and is not visible anymore. (D–D''') At stage NS11 all neuroblasts have delaminated forming a pattern of seven rows and up to five columns. The MP1 is not longer detectable. For abbreviations see abbreviation index page 10; segment borders are indicated by horizontal dashed lines; the midline is indicated by a vertical dashed line; scale bar = 50 µm (A-D); 10 µm (A'-D'').

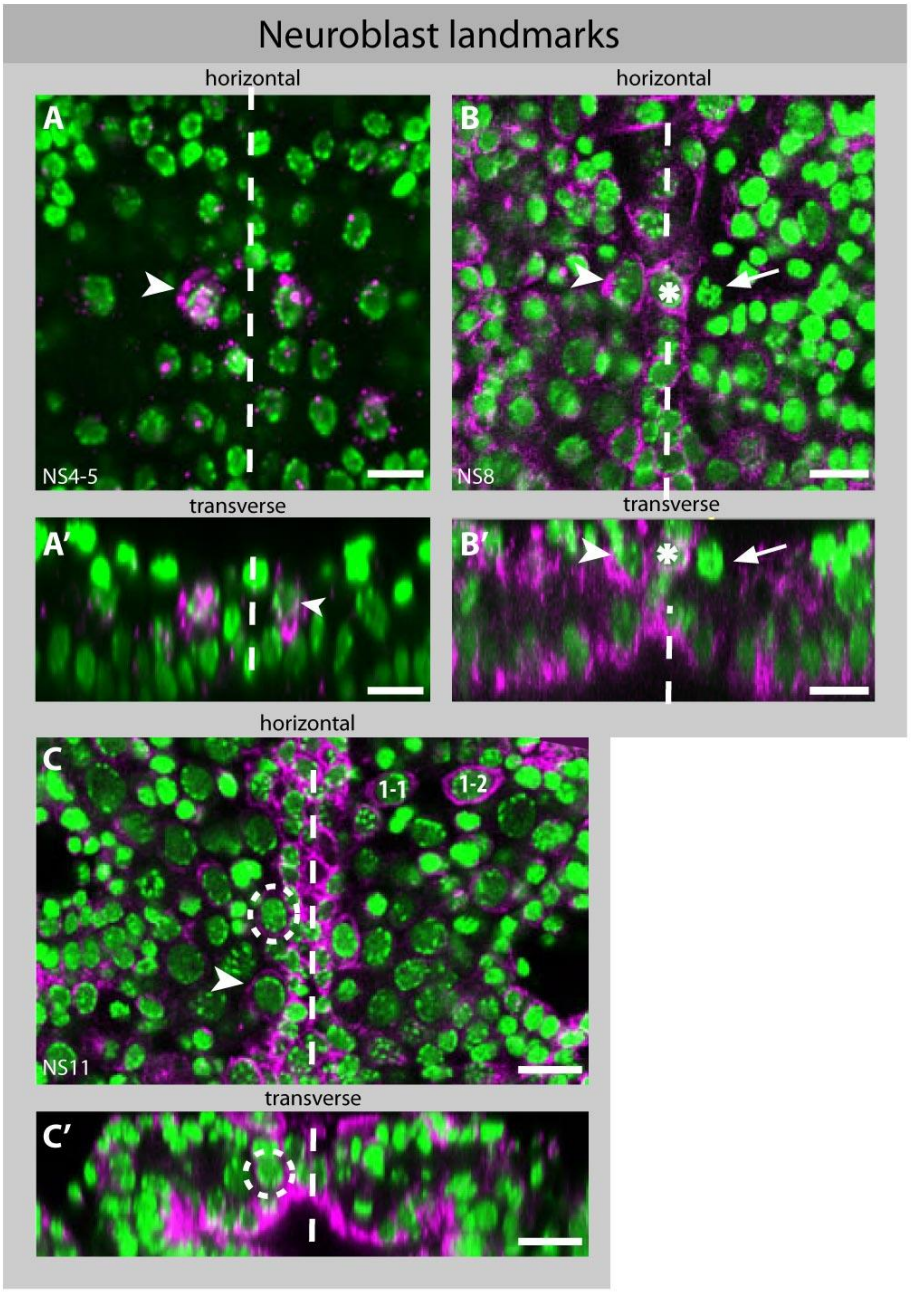


Figure 3-1.5: Neuroblast landmarks

Horizontal and transverse sections of different *Tribolium* stages showing the MP2, MP1, NBs 5-1 and 6-1. Hoechst nuclei stain (green); α-tubulin labeling of cell membranes (magenta) (B, B', C, C'). *Tc-pros* RNA is detected by fluorescent *in situ* hybridisation (A, A'). Anterior is towards the top (A, B, C). Basal is to the top (A', B', C'). (A) MP2 is the first cell in in the neuroblast layer expressing *Tc-pros* (arrowhead). (B, B') MP2 is recognizable by its elongated appearance and position close to the midline on both sides of the midline (arrowhead). The single MP1 is positioned between the two MP2s of one segment (asterisk). Note MP2 in right hemineuromere is in mitosis (arrow). (C, C') NB 5-1 (circle) delaminates close to the midline during NS11. NB 6-1 (arrowhead) which already delaminates during NS8 is positioned close to the midline (arrowhead). The midline is indicated by a vertical dashed line; scale bar = 10 µm.

Construction of a *Tribolium* neuroblast map on the basis of orthologues of *Drosophila* neuroblast identity genes

The neuroblast map obtained, using morphological data, can now be utilised as a tool to conduct thorough comparisons of gene expression patterns between *Drosophila* and *Tribolium*. The following section aims to provide a neuroblast map comparable to that for *Drosophila*, developed by Doe (1992) and refined by Broadus *et al.* (1995) (Fig. 1-2). Additional *Drosophila* neuroblast specific genes have since been analysed but not added to the neuroblast map of Doe (1992). *Tribolium* orthologues of the neural identity genes of *Drosophila* were cloned and their expression pattern was visualised via colorimetric and fluorescent *in situ* hybridisation. Some of these genes have previously been analysed concerning their role in segmentation in *Tribolium*, but not in the nervous system, and others have not been described at all. All genes except for *Tc-mirror* were successfully cloned using specific primers but the respective probes produced results of varying quality when used for *in situ* hybridisation.

The first set of *Tribolium* orthologues that will be described are the segment polarity genes *engrailed* (*Tc-en*), *wingless* (*Tc-wg*) and *gooseberry distal* (*Tc-gsb*). In *Drosophila* these genes are expressed along the AP axis in one to three rows of neuroblasts (see review Bath 1999). Unlike the segment polarity genes, the expression of the following genes described – *huckebein, runt, seven-up* (*Tc-hkb, Tc-run, Tc-svp*) – is not restricted to specific rows. The expression patterns are characterised by transient expression. The last group of genes analysed are the temporal genes *hunchback*, *Krüppel*, *nubbin* and *castor* (*Tc-hb, Tc-Kr, Tc-nub, Tc-cas)*. In *Drosophila* they are expressed in a timely manner.

Expression of segment polarity genes in the neuroectoderm and neuroblasts

Tc-engrailed expression

The expression of the segmentation gene *engrailed* is highly conserved between different species of arthropods (Patel *et al.* 1989). Its expression pattern has been thoroughly analysed in *Tribolium* and expression in neuronal cells has been described (Brown *et al.* 1994a). *Tc-en* expression has, however, not been assigned to specific neuroblasts.

Expression of *Tc-en* in *Tribolium* neuroblasts was analysed by *in situ* hybridisation. *Tc-en* is expressed in ectodermal cells in the posterior part of each *Tribolium* segment (Fig. 3-1.6 A, B, C). It is expressed in neuroblasts of row six, seven and the MNB in *Tribolium* (Fig. 3-1.6 D, E, F). According to the established map there is no expression in row one neuroblasts which stands in contrast to *Drosophila* (Fig. 3-1.6 E, F).

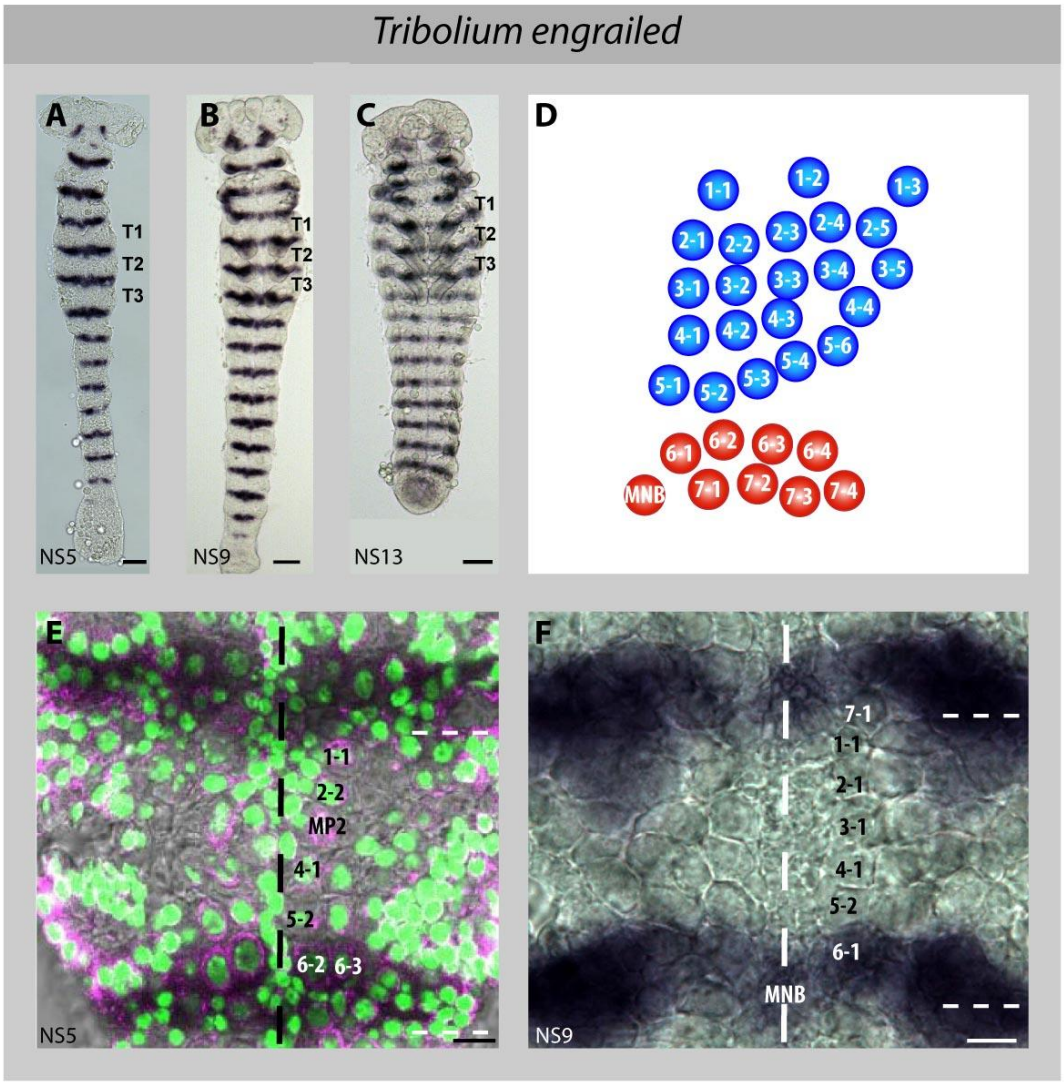


Figure 3-1.6: *Tc-en* expression in neuroblasts

Tc-en expression detected in embryos by *in situ* hybridisation. Anterior is towards the top. (A-C) *Tc-en* is expressed in the posterior part of every segment. (D) Schematic neuroblast map of a single hemineuromere. *Tc-en* is expressed in all row six and seven neuroblasts (red). (E) Confocal image of the first thoracic segment of the embryo shown in A, combining bright field *Tc-en* expression (black), Hoechst staining in nuclei (green) and α-tubulin staining (magenta). Ectodermal cells and neuroblasts of row six and seven express *Tc-en.* (F) First thoracic segment of the embryo in B, showing neuroblast 6-1 and the MNB expressing *Tc-en.* For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line, segment borders are indicated by horizontal dashed lines; scale bar = 50 µm (A-C), 10µm (E, F).

Tc-wingless expression

The expression pattern of the segment polarity gene *wingless* during segmentation appears to be conserved between *Drosophila* and *Tribolium* (Nagy and Carroll 1994). The expression of *Tc-wg* in the ventral nervous system of *Tribolium* has not been analysed to date. *Tc-wg* mRNA is detected in a two to three cell wide stripe of ectodermal cells in a repeated pattern in the posterior part of all segments in stage NS4 *Tribolium* embryos (Fig. 3-1.7 A). Noticeable is a decrease of *Tc-wg* expression towards the midline, with no expression in midline cells (Fig. 3-1.7 A'). Three neuroblasts of which NBs 5-2 and 5-3 can be identified arise from the *Tc-wg* positive neuroectoderm and express *Tc-wg* initially. Additionally a neuroblast lateral to NB 5-3 expresses *Tc-wg*. This neuroblast is most likely NB 5-4, though there is also a possibility that it may be NB 5-6. Neuroblast 5-2 delaminates closest to the midline, out of an area of weak *Tc-wg* expression. *Tc-wg* expression in NB 5-2 is very weak and only lasts for a short period of time (Fig. 3-1.7 B'). The expression of *Tc-wg* in the neuroectoderm ceases concurrently with the delamination of neuroblasts. Between stage NS11 and NS12 *Tc-wg* expression is only detected in NB 5-4 (Fig. 3-1.7 C'). Additionally, *Tc-wg* expression is detectable in the legs (Fig. 3-1.7 C''). This expression can easily be mistaken for expression in neuroblasts. From stage NS12 onwards no expression occurs in neural cells in the thoracic segments (Fig. 3-1.7 D, E, E'). In abdominal hemineuromeres, however, expression persists in neural cells until stage NS13 (Fig. 3-1.7 D, E).The later formed NB 5-1 and the lateral putative NB 5-6 do not express *Tc-wg*.

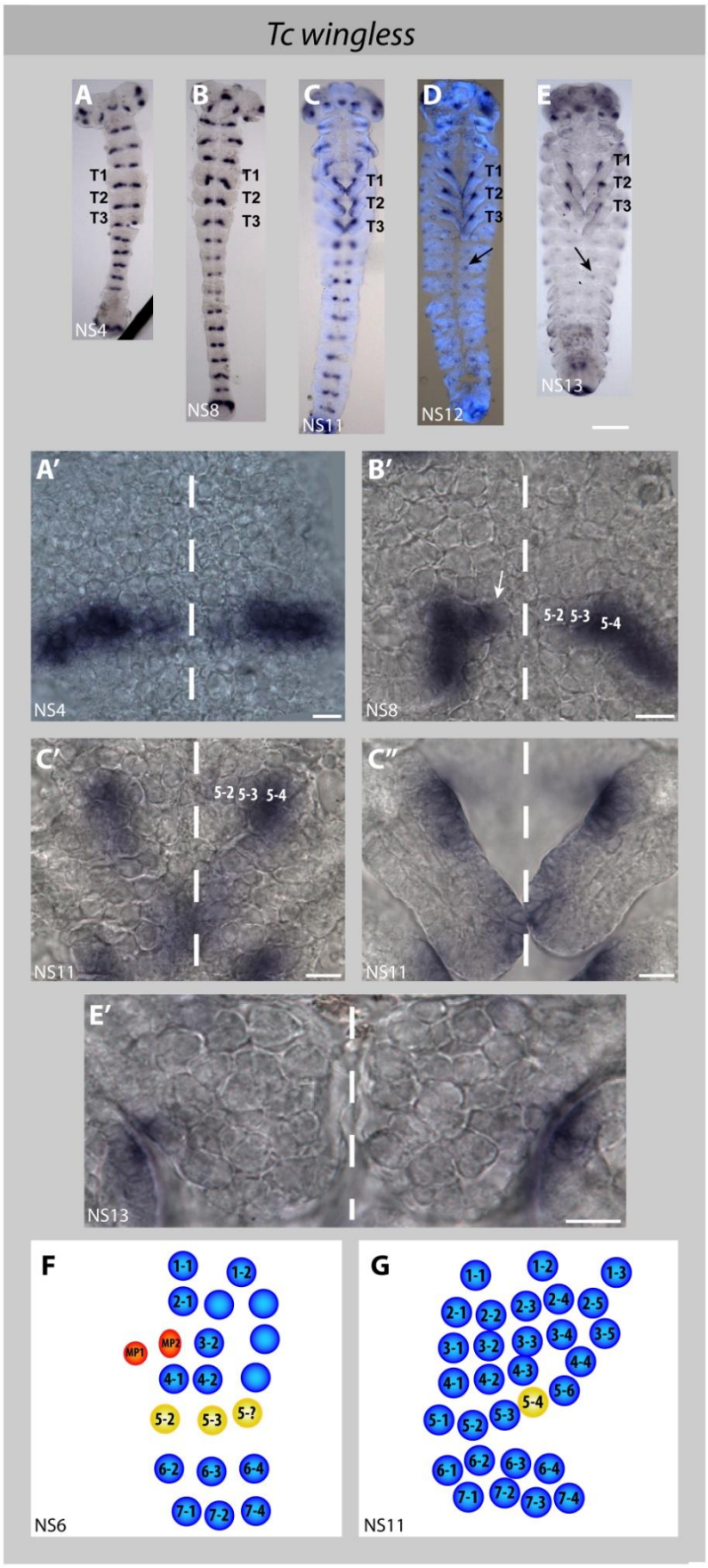


Figure 3-1.7: *Tc-wg* expression in the nervous system of *Tribolium*

Figure 3-1.7: *Tc-wg* expression in the nervous system of *Tribolium*

Tc-wg expression in *Tribolium* embryos detected by *in situ* hybridisation (A-E). Additionally, higher magnification images of the first thoracic segment of embryo A, B, C and E are shown (A', B', C'', E'). Anterior is towards the top. (A–E) *Tc-wg* is expressed in ectodermal cells in stage NS4. Expression in thoracic segments is eventually restricted to neuroblasts. From stage NS12 onwards there is no expression detectable in neuroblasts in the thoracic segments. There is, however, weak expression remaining in neural cells of the abdominal segments (D, E, arrow). (A') Higher expression is visible in a two to three cell wide row of ectodermal cells in the posterior part of the hemineuromere. (B') Row five neuroblasts delaminate out of the *Tc-wg* expressing row of ectodermal cells. Neuroblast 5-2 appears to express *Tc-wg* transiently (hemineuromere at left side of figure B', arrow). Additionally, NB 5-3 and NB 5-4 express *Tc-wg.* (C') The expression of *Tc-wg* in NBs 5-2 and 5-3 ceases. At stage NS11 only NB 5-4 is detectecd expressing *Tc-wg*. (C'') The same segment shown in C, with focus on the ventral side of the embryo, thereby visualising *Tc-wg* expression in the appendages. (E') There is no expression of *Tc-wg* detectable in neuroblasts or neurons in the hemineuromere, but expression is detectable in the associated appendage. (F) Schematic drawing of NS6 neuroblast map showing the expression of *Tc-wg* in NBs 5-2 and 5-3 and 5-? in yellow. Note, that stage NS6 is only shown as a schematic drawing with no corresponding *in situ* hybridisation presented. (G) Schematic drawing of NS11 neuroblast map depicting *Tc-wg* expression in NB 5-4. For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line; segment borders by the horizontal dashed lines; scale bar = 100 µm (A-E), 10µm (A'-E').

Tc-gooseberry expression

The segment polarity gene *gooseberry* is expressed in ectodermal cells and neuroblasts of row five and six in *Drosophila* (Gutjahr *et al.* 1993). In *Tribolium Tc-gsb* expression in the nervous system is first detected in ectodermal cells along the posterior area of a hemineuromere out of which neuroblasts of row five and six arise (Fig 3-1.8 A, A'). All neuroblasts of row five and six express *Tc-gsb* (Fig. 3-1.8 B', C', F). The expression appears to last from the time of their formation in the ectoderm until the end of their lifespan. Additionally, *Tc-gsb* expression in the ectoderm persists even after neuroblasts have delaminated (Fig 3-1.8 B''). Beyond that GMCs and neurons of row five and six neuroblasts express *Tc-gsb* (Fig 3-1.8 C''). At stage NS15 *Tc-gsb* expression is additionally detected in the tracheal system (Fig 3-1.8 E, E').

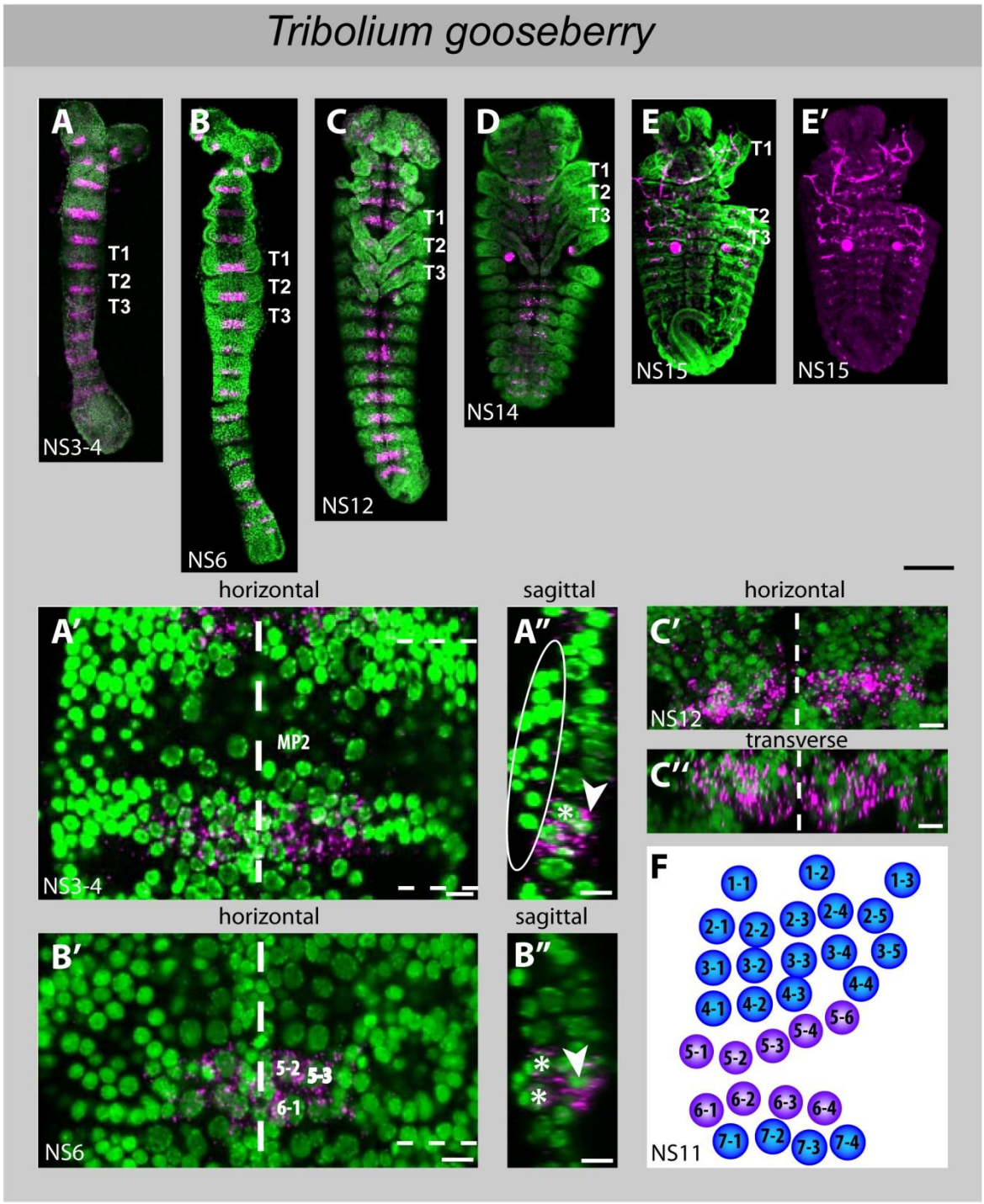


Figure 3-1.8: *Tc-gsb* expression in the nervous system of *Tribolium*

Figure 3-1.8: *Tc-gsb* expression in the nervous system of *Tribolium*

Tc-gsb mRNA expression detected in embryos by fluorescent *in situ* hybridisation (magenta) (A-E'). Additionally, T1 segments of A, B and C are shown at higher magnification (A', B', C'); nuclei staining (green); Imaris full volume (A-E') and section mode (A', A'', B', B'', C', C''). Anterior is towards the top. Basal is to the left (A'', B'') and to the top (C''). (A-D) *Tc-gsb* is expressed in a band several cells wide along the posterior border of all segments in successive stages of *Tribolium.* (E, E') At stage NS15 expression of *Tc-gsb* is detected in neurons and axons in the gnathal, thoracic segments and the head. (A') Horizontal section of T1 of embryo shown in A. *Tc-gsb* is detected in a three to four cell wide band of ectodermal cells. (A'') Sagittal section of A'. *Tc-gsb* expressing neuroblasts are in the process of delaminating inside the embryo (asterisk). Ectodermal cells apical to them express *Tc-gsb* (arrowhead). Cells basal to them are mesodermal cells (white oblong). (B') Horizontal section of T1 of embryo shown in B. Neuroblasts 5-2, 5-3, 6-1 and 6-2 express *Tc-gsb.* (B'') Sagittal section of B'. Neuroectodermal cells (arrowhead) apical to delaminated neuroblasts (asterisk) express *Tc-gsb.* (C') Horizontal section of embryo shown in C, *Tc-gsb* expression is still detected in neuroblasts and progenitor cells at stage NS12. (C'') Transverse section of C'. *Tc-gsb* is expressed along the whole apical basal axis in neuroblasts and their progenitor cells. (F) Schematic neuroblast map of stage NS11 depicting all neuroblasts expressing *Tc-gsb.* Note, that there is no corresponding *in situ* hybridisation presented. For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line; segment borders are indicated by horizontal dashed lines; scale bar = 100 µm (A-E'), 10 µm (A'-C'').

Tc-huckebein expression

Prior to this work there were no data available concerning *huckebein* expression in the nervous system of *Tribolium*. The first cells expressing *Tc-hkb* in the nervous system occur around stage NS2 in the middle of the maxillary segment (Fig. 3-1.9 A). Subsequently the mandibular, labial and first two thoracic segments begin to express *Tc-hkb* in a cluster of ectodermal cells in the middle of the respective segment, corresponding to row four. Additionally, cells in the head lobes start expressing *Tc-hkb* (Fig. 3-1.9 B). At stage NS5 the first neuroblasts expressing *Tc-hkb* are detected as NB 4-2 and a row four neuroblast laterally to it, followed by NBs 5-3 and 2-2 (Fig. 3-1.9 C, C'). Expression in a further later neuroblast of row four and NB 1-1 follows at stage NS9 (Fig. 3-1.9 D'). It is not possible to unequivocally determine wether the third row four neuroblast delaminates between neuroblast 4-2 and the additional row four neuroblast or lateral to the two neuroblasts. Therefore neuroblast identities can only be designated from stage NS8 onwards (Fig. 3-1.9 D', I). It should be further noted that there is considerable variation between different embryos, at a similar developmental stage, regarding which neuroblasts express *Tc-hkb.* In some embryos there is also expression in ectodermal cells of row seven and ectodermal cells laterally to NB 1-1 (Fig. 3-1.9 D'). There is, however, no expression detectable in row seven neuroblasts or additional row one neuroblasts. *Tc-hkb* expression in stage NS11 embryos is decreasing, although a strong expression persists in NB 1-1 (Fig. 3-1.9 E). In stage NS13 a single neuroblast at the anterior edge of the hemineuromere and a cluster of neurons express *Tc-hkb*, probably NB 1-1 (Fig. 3-1.9 F, F'). Expression in neurons appears to persist in the last stage analysed (NS15; Fig. 3-1.9 G).

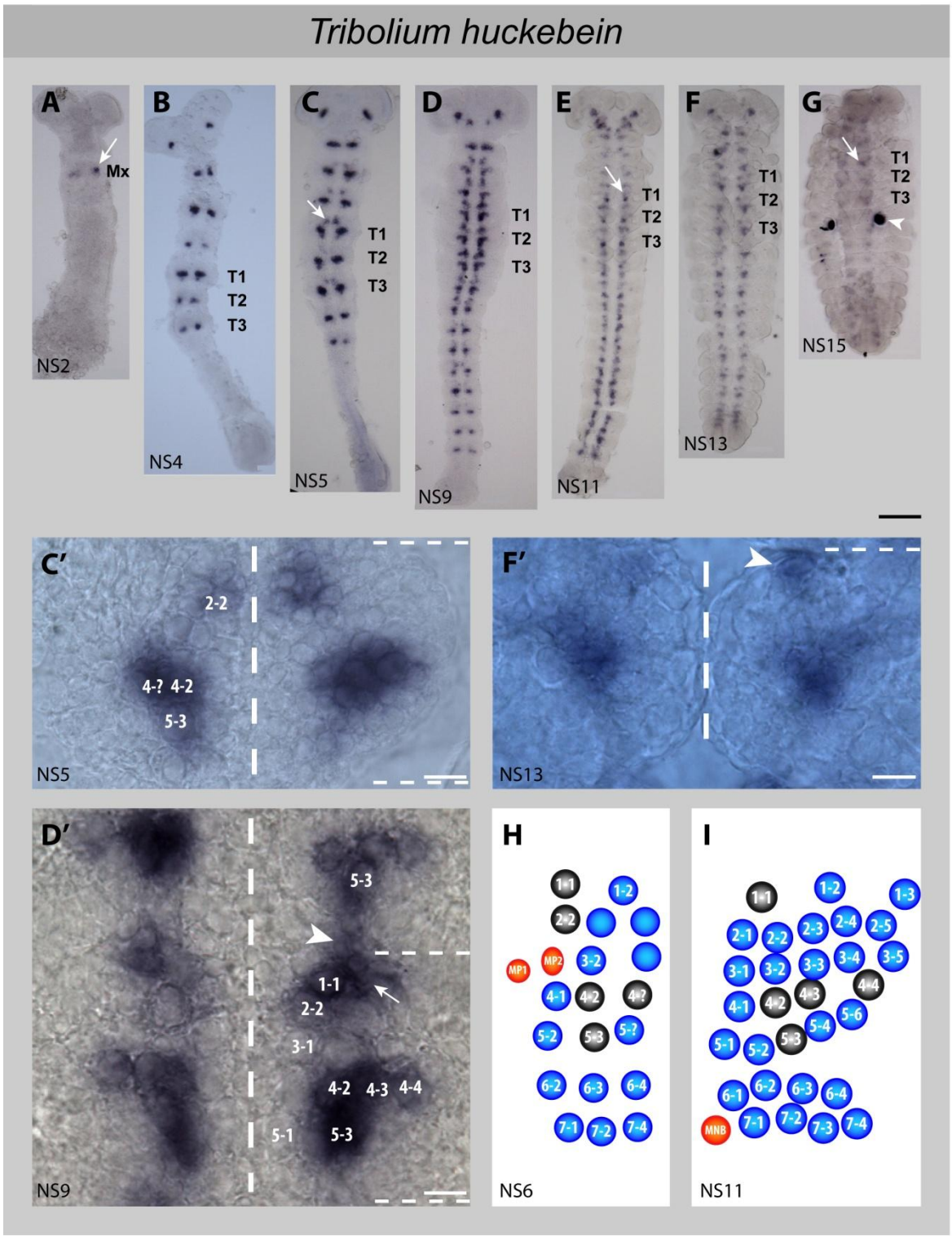


Figure 3-1.9: *Tc-hkb* expression in the nervous system of *Tribolium*

Figure 3-1.9: *Tc-hkb* expression in the nervous system of *Tribolium*

Tc-hkb mRNA expression detected in embryos (A-G) and in T1 segments of C, D and F by *in situ* hybridisation (C', D', F'). Anterior is towards the top. (A) *Tc-hkb* expression is first detected in a cell in the maxillary segment at stage NS2 (arrow). (B) The expression of *Tc-hkb* extends over the remaining gnathal and thoracic segments and further neuroectodermal cells begin to express *Tc-hkb* at stage NS4. (C, D) Over the following stages a second cluster of cells expressing *Tc-hkb* appears (arrow). (E) At stage NS11 *Tc-hkb* expression decreases with NB 1-1 still strongly expressing it (arrow). (F) The expression of *Tc-hkb* further decreases in Stage NS13. (G) In stage NS15 embryos weak expression in neurons is still detectable (arrow). Note background staining in pleuropodia (arrowhead). (C') *Tc-hkb* is expressed in NBs 2-2, 4-2, 5-3 and a further row four neuroblast. (D') Additional expression is detected in NB 1-1 and NB 4-4. Furthermore, expression in ectodermal cells of row seven (arrowhead) and laterally to NB 1-1 (arrow) is detected. (F') Expression of *Tc-hkb* is detectable in one neuroblast, probably NB 1-1 (arrowhead) and a cluster of neurons. (H, I) Schematic drawings with no corresponding *in situ* hybridisation presented. (H) Schematic drawing of neuroblast map at stage NS6 showing the expression of *Tc-hkb* in black. (I) Schematic drawing of neuroblast map at stage NS11. For abbreviations see abbreviation index; the midline is indicated by a vertical dashed line, segment borders by horizontal dashed lines; scale bar = 100 µm (A-G), 10µm (C', D' F').

Tc-runt expression

The pair rule gene *Tc-runt* has been analysed regarding its role during segmentation in *Tribolium* (Brown and Denell 1996). The following is the first description of its expression in the nervous system of *Tribolium. Tc-run* expression in the nervous system first occurs between stage NS2 and NS3 in neuroectodermal cells in the gnathal segments (Fig. 3-1.10 A). The expression expands, resulting in two distinct *Tc-run* expressing ectodermal cell clusters per hemineuromere by stage NS6. One large cluster is positioned in the medial and intermediate column neuroectoderm, in the anterior part of a segment, partially extending over the midline. The second cluster comprises three to four cells and lies posteriorly in the medial neuroectoderm (Fig. 3-1.10 B, F). Basal to the *Tc-run* expressing ectodermal cells, neuroblasts expressing *Tc-run* are detected (Fig. 3-1.10 F'). At stage NS6 *Tc-run* expression can be assigned to NBs 2-2, 3-2 and 6-2. Additionally, *Tc-run* is expressed in two more neuroblasts in row three and one more neuroblast in row two (Fig. 3-1.10 F'). The row two neuroblast is very likely NB 2-3. The row three neuroblasts could be either NBs 3-3 and 3-4 or NBs 3-3 and 3-5. Furthermore, it is possible that *Tc-run* is expressed in MP2 and MP1 but to verify this, a fluorescent *in situ* hybridisation would have to be performed. *Tc-run* expression in the three thoracic segments of an embryo two to three stages later in development reveals a decrease of

Tc-run expression. Neuroblasts 6-2, 2-2 and 3-2 maintain *Tc-run* expression, whilst the possibel NBs 3-3, 3-4/3-5 and the possible NB 2-3 cease the expression. Futhermore NB 2-1, which does not express *Tc-run* has delaminated between NB 2-2 and the midline visible in the second thoracic segment. Additionally, there is no expression in the position of MP1 and MP2 detectable. (Fig. 3-1.10 G). At stage NS13 *Tc-run* expression is detectable in neurons covering almost the entire hemineuromere (Fig. 3-1.10 D, H).

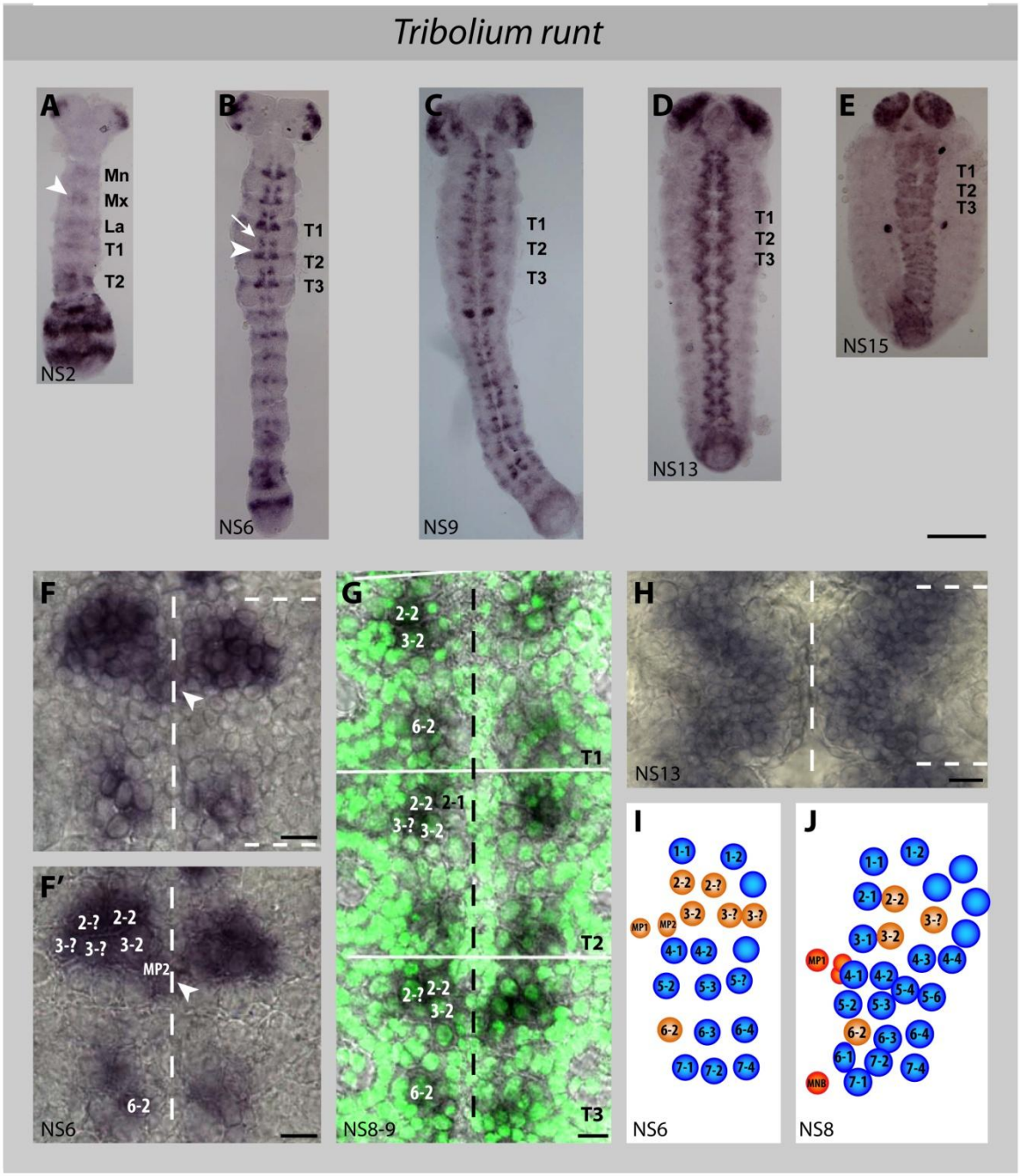


Figure 3-1.10: *Tc-runt* is expressed in row two,three and six neuroblasts

Figure 3-1.10: *Tc-runt* is expressed in row two, three and six neuroblasts

Tc-run mRNA expression detected by *in situ* hybridisation in *Tribolium* embryos (A-E), T1 segments (F, F', H) and in T1, T2, T3 (G). Anterior is towards the top. (A) Expression of *Tc-run* in the nervous system first appears at stage NS2 in the gnathal segments (arrowhead). (B) At stage NS6 ectodermal cells and neuroblasts express *Tc-run* in the anterior (arrow) and posterior (arrowhead) part of a hemineuromere. (C) The expression broadens slightly over time. (D) At stage NS13 many neurons expressing *Tc-runt* are detected. (E) At stage NS15 the expression in neurons decreases. (F) Expression of *Tc-run* in ectodermal cells in the first thoracic segment of embryo shown in B. There is a large cell cluster in the anterior part of the hemineuromere extending over the midline (arrowhead) and three to four ectodermal cells in the posterior part. (F') Basal view of hemineuromere in F, showing the expression of *Tc-run* in neuroblasts of row two, three and NB 6-2. Additionally, the midline precursor cells MP2 and MP1 appear to express *Tc-run* (arrowhead). (G) Superimposition of a confocal image with cell nuclei in green (Hoechst) and *in situ* staining in black. In an embryo two to three stages older the expression becomes restricted to neuroblasts 2-2 and 3-2 (T1) (H) First thoracic segment of embryo shown in D. The expression of *Tc-run* continues in neurons along most of the hemineuromere. (I) Schematic neuroblast map at stage NS6 based on expression pattern in F'. (J) Schematic neuroblast map of one hemineuromere at stage NS8. For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line, segment borders by horizontal white dashed or white solid lines in G; scale bar = 100 µm (A-E), 10µm (F-H).

Tc-seven-up expression

The nuclear receptor *seven-up* (*svp*) is expressed in almost all neuroblasts in *Drosophila* and acts as a regulator of temporal gene expression. It is expressed transiently and therefore only has an effect on a subsection of each neuroblast lineage (Doe 1992, Kanai 2005). Presently, no data exist regarding *seven-up* expression in *Tribolium*. As a consequence of difficulties in generating *in situ* hybridisation data for this gene, a complete analysis of *Tc-svp* expression in the ventral nervous system of *Tribolium* was not possible. Nonetheless, the first neuroblast expressing *Tc-svp* in *Tribolium* can be identified as NB 5-2, at around stage NS5 (Fig. 3-1.11 A, A'). Three stages later, at stage NS8, row three, four and five neuroblasts express *Tc-svp* (Fig. 3-1.11 B'). Around stage NS10 the expression decreases (Fig 3-1.11 C'). As in *Drosophila* the expression pattern of *Tc-svp* appears to be dynamic, with not all neuroblasts expressing it at the same time (compare Fig. 3-1.11 B' and C'). *Tc-svp* expression in the peripheral nervous system is detectable around stage NS12, whilst expression in the ventral nervous system ceases around this stage (Fig. 3-1.11 D). By stage N15, cells positioned laterally at the posterior and anterior edge of a hemineuromere express *Tc-svp* (Fig. 3-1.11 E, E'). These cells are likely to be neurons.

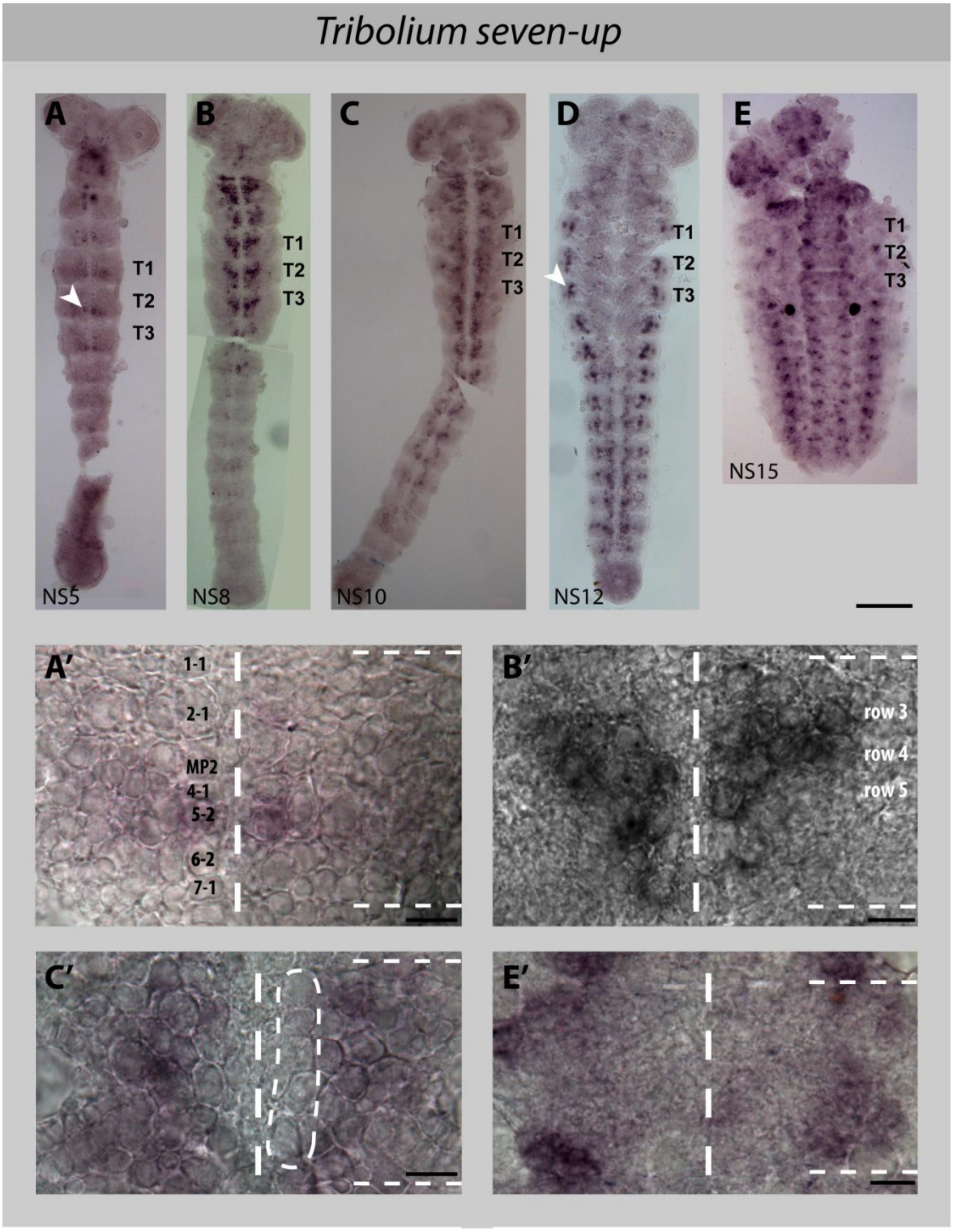


Figure 3-1.11: *Tc-svp* expression in the nervous system of *Tribolium*

Figure 3-1.11: *Tc-svp* expression in the nervous system *Tribolium*

Tc-svp expression detected in embryos by *in situ* hybridisation (A-E). (A', B', C', E') Higher magnification images of the first thoracic segment of the respective embryos from A, B, C and E. Anterior is towards the top. (A, A') The first cell to express *Tc-svp* in the nervous system is NB 5-2 (arrowhead). (B, B') At stage NS8 expression has extended to neuroblasts of row three, four and five. (C, C') Expression at stage NS10 is very weak, with no *Tc-svp* expression in ventral neuroblasts (dashed oblong). (D) At stage NS12 expression has ceased in neuroblasts of the thoracic segments. There is, however, expression detectable in the peripheral nervous system (arrowhead). (E, E') Expression in the nervous system is detectable again at stage NS15 in neurons in the anterior and posterior region of a hemineuromere. For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line, segment borders by horizontal dashed lines; scale bar = 100 µm (A-E), 10µm (A'-E').

Temporal gene expression in *Tribolium*

hunchback, *Krüppel*, *pdm* and *castor* are sequentially expressed in almost all neuroblasts of *Drosophila* (Isshiki *et al.* 2001). *Kr* and *hb* are additionally involved in segmentation processes in *Drosophila* and have been shown to have a similar role during segmentation in *Tribolium* (Schroeder 2003, Wolff *et al.* 2005). There is, however, no information on their function during neurogenesis. *castor and nubbin* expression have not been analysed during *Tribolium* embryogenesis.

Tc-hunchback

In *Tribolium*, as in *Drosophila*, *hunchback* is the first temporal gene to be expressed in neuroblasts (Fig. 3-1.12 A). It is first detected in ectodermal cells along a whole segment (Fig 3-1.12 A1). Coinciding with the start of neuroblast delamination, the expression of *Tc-hb* becomes restricted to ectoderm cells and neuroblasts (Fig. 3-1.12 A2; Fig. 3-1.13 A2'). Almost all neuroblasts appear to express *Tc-hb* at some point in nervous system development. However, this expression is only temporary and therefore not all neuroblasts express *Tc-hb* at the same time. *Tc-hb* expression ceases around stage NS15 (Fig. 3-12 A7)

Tc-Krüppel

The gap gene *Krüppel* is the second gene to be expressed in the temporal gene expression cascade (Fig. 3-1.12 B). In its function as a gap gene it is strongly expressed in whole segments in young embryos (Fig. 3-1.12 B1). Its expression in the nervous system is rather diffuse, with almost all ectodermal cells expressing it (Fig. 3-1.12 B2, B3; Fig. 3-1.13 B2', B3'). In later stages single neuroblasts can be identified but not assigned to specific neuroblast identities (Fig. 3-1.12 B4; Fig. 3-1.13 B3', B4', B5'). The expression of *Tc-Kr* appears to decrease before *Tc-hkb* expression does. (compare Fig. 3-1.12 A5 + B5, Fig. 3-1.13 A5' + B5').

Tc-nubbin

Tc-nubbin (known as *pdm1* in *Drosophila*) is expressed in the growth zone and the distal tip of the appendages before weak expression is visible in the neuroectoderm around stage NS6 (Fig. 3-1.12 C 3; Fig. 3-1.13 C3'). The first neuroblasts expressing *Tc-nub* appear between stage NS7 and NS8 along the ventral column of the neuroectoderm (Fig. 3-1.12 C4; Fig. 3-1.13 C4'). *Tc-nub* expression is detected in most neuroblasts at some point. Additionally, strong expression persists in neurons even in the last stage of neurogenesis analysed (NS15) (Fig. 3-1.12 C7).

Tc-castor

The transcription factor *castor* (also known as *ming* in *Drosophila*) is the last of the temporal genes to be expressed in neuroblasts (Fig. 3-1.12 D). *Tc-cas* expression begins in the anterior region of the growth zone at stage NS4 (Fig. 3-1.12 D2). At stage NS6 expression is detected in two cells in the mandibular segment, left and right to the midline. Additionally *Tc-cas* expression occurs in cells in the labrum and two cell clusters along the midline between the maxilllary and labial segment and between the labial and first thoracic segment (Fig. 3-1.12 D3; Fig. 3-1.13 D3'). Over the next two stages the expression extends to distinct tissues in the head lobes, the tips of the appendages and midline cell clusters between adjacent segments in thoracic and abdominal segments. Subsequently, the first neuroblasts start expressing *Tc-cas* around stage NS8 (Fig. 3-1.12 D4; Fig. 3-1.13 D4'). The early expression pattern in neuroblasts is reminiscent of a necklace with ventral column neuroblasts expressing *Tc-cas* (Fig. 3-1.12 D4; Fig. 3-1.13 D'). The expression from there appears to extend over most neuroblasts (Fig. 3-1.12 D5; Fig. 3-1.13 D5'). Additionally, expression in the peripheral nervous system is detected around stage NS14 (Fig. 3-1.13 D6). A diffuse expression in neurons persists till stage NS15 (Fig. 3-1.12 D7).

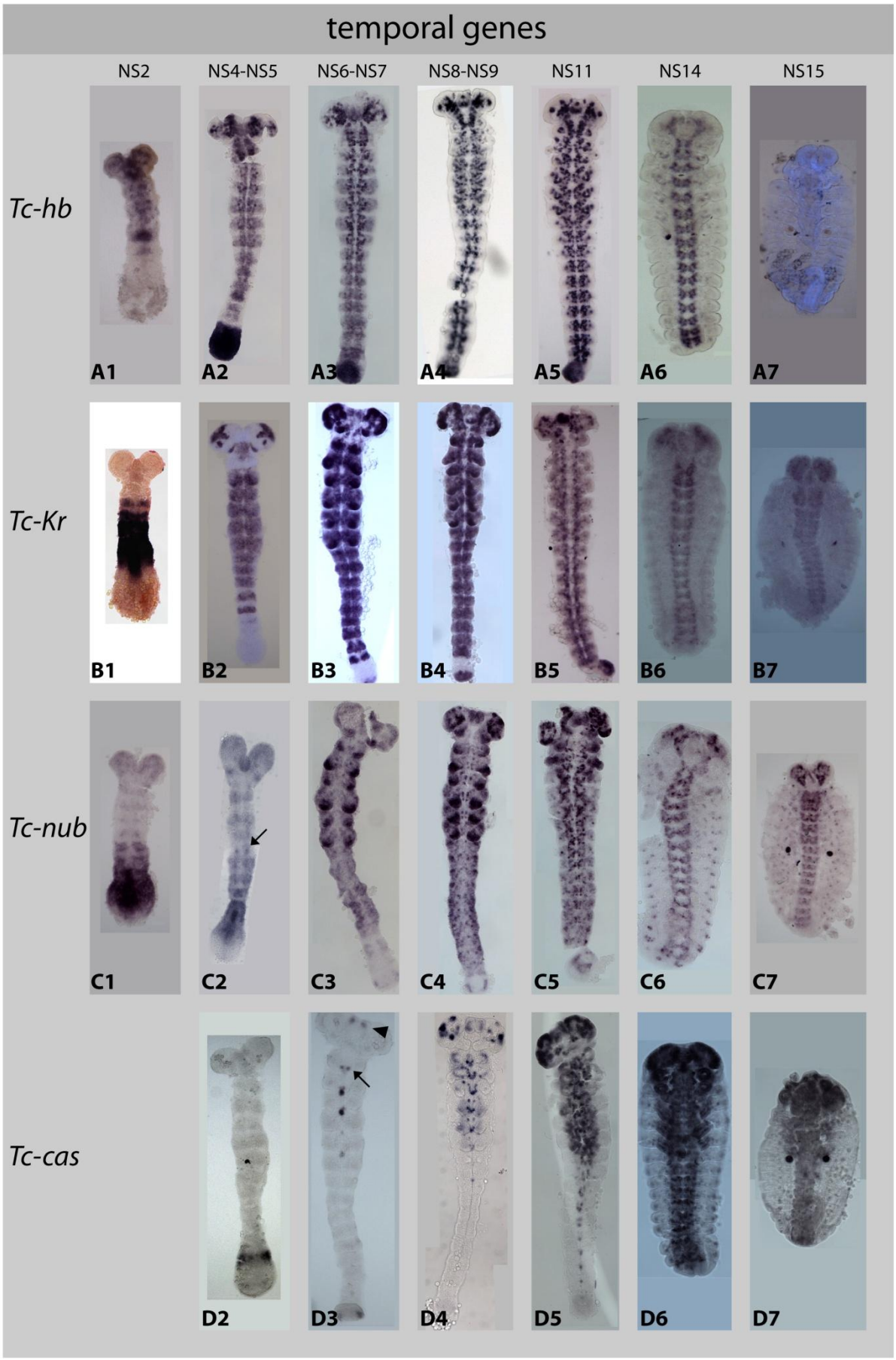


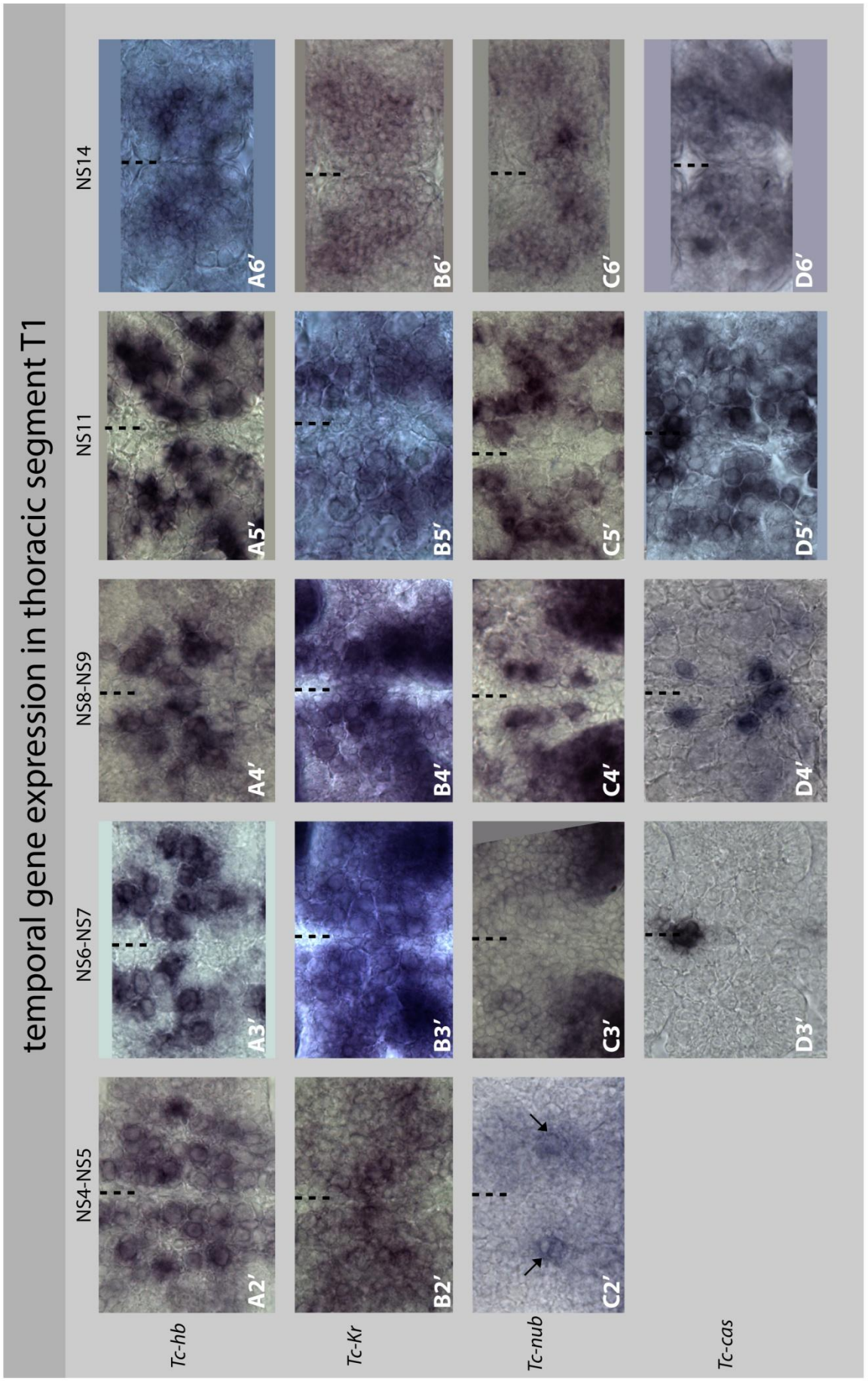
Figure 3-1.12: Temporal genes are expressed sequentially in *Tribolium*

Figure 3-1.12: Temporal genes are expressed sequentially in *Tribolium*

Tc-hb, Tc-Kr, Tc-nub, Tc-cas mRNA expression in successive stages (NS2, NS4-5, NS6-7, NS8-9, NS11, NS14, NS15) of embryos (A, B, C, D respectively) detected by *in situ* hybridisation. Anterior is to the top. (A1) *Tc-hb* expression starts at stage NS2 in ectodermal cells in the neuroectoderm. (A2) With the formation of neuroblasts *Tc-hb* expression becomes restricted to neuroblasts. (A3-A5) All neuroblasts appear to express *Tc-hb* at a certain time during their life span. (A6) Some neuroblasts and neurons are left expressing *Tc-hb.* (A7) At stage NS15 there is no *Tc-hb* expression detectable in any part of the embryo. (B1) *Tc-Kr* is very strongly expressed in the neuroectoderm at stage NS2. (B2, B3) Ectodermal cells continue *Tc-Kr* expression with some neuroblasts additionally commencing its expression. (B4, B5) Expression becomes restricted to neuroblasts. (B6) *Tc-Kr* expression is detectable in neurons. (B7) Little to no *Tc-Kr* expression is detectable by stage N15. (C1) At stage NS2 *Tc-nub* expression is detected in the growth zone and abdominal segments. (C2) Faint expression of *Tc-nub* is detectable in all segments of the embryo, not specifically in the neuroectoderm other than for one or two cells (arrow). (C3) At stage NS6 expression in the appendages and lateral neuroectoderm appears. (C4) The first neuroblasts along the midline commence the expression of *Tc-nub* at stage NS8. *Tc-nub* expression in the appendages increases. (C5) Increasing numbers of neuroblasts are detected expressing *Tc-nub*. (C6) Neurons and some neuroblasts are detected expressing *Tc-nub* at stage NS14. (C7) The expression persists in neurons till the last stage analysed (NS15). (D2) At stage NS4 *Tc-cas* is expressed in a stripe in the growth zone. There is no expression in the neuroectoderm. (D3) A large cell cluster along the midline, some cells in the labrum (arrowhead) and two cells in the mandibular segment (arrow) express *Tc-cas.* (D4) Ventral neuroblasts strongly express *Tc-cas* at stage NS8. Additional expression in the head and appendages is detectable. (D5) Increasing numbers of neuroblasts start to express *Tc-cas.* (D6) At stage NS14 *Tc-cas* expression is detectable in neurons and neuroblasts. (D7) There is no expression remaining at stage NS15.

Figure 3-1.13: Temporal gene expression in first thoracic segments of successive *Tribolium* stages

Tc-hb, Tc-Kr, Tc-nub, Tc-cas mRNA expression in successive stages (NS4-5, NS6-7, NS8-9, NS11, NS14), of the first thoracic segments (A', B', C', D') of the embryos presented in Fig. 3-1.13, detected by *in situ* hybridisation. Anterior is to the left. (A2') *Tc-hb* expression in distinct neuroblasts in roughly three columns. (A3') Expression extends laterally with some neuroblasts ceasing *Tc-hb* expression and others commencing *Tc-hb* expression. (A4', A5') Some additional neuroblasts to those identified earlier appear to express *Tc-hb.* (A6') At stage NS14 expression is only detected in neurons. (B2') *Tc-Kr* is expressed in ectodermal cells and some neuroblasts. (B3') *Tc-Kr* is expressed uniformly in almost all neuroblasts. (B4') Differences in intensity of *Tc-Kr* expression between neuroblasts is observable. (B5') *Tc-Kr* expression becomes restricted to a few neuroblasts. (B6') At stage NS14 *Tc-Kr* is expressed in neurons. (C2') *Tc-nub* expression is first detected in the neuroectoderm at stage NS4-5 in one to two ectodermal cells per hemisegment (arrow). (C3') Over time the expression of *Tc-nub* broadens over several ectodermal cells. (C4') At stage NS8 ventral neuroblasts express *Tc-nub.* (C5') The expression extends over many neuroblasts. (C6') At stage NS14 faint expression of *Tc-nub* is detected in neurons. (D3') *Tc-cas* expression in the neuroectoderm only commences at stage NS6 in a cluster of midline cells. (D4') The expression expands to ventral neuroblasts. (D5') Eventually many neuroblasts appear to express *Tc-cas* by around stage 11. (D6') At stage 14 neurons and some neuroblasts are detected expressing *Tc-cas.* The dashed line indicates the midline.


temporal gene expression in thoracic segment T1
NS4-NS5
NS6-NS7
NS8-NS9
NS11
NS14
Tc-hb
Tc-Kr
Tc-nub
Tc-cas
A2′
A3′
A4′
A5′
A6′
B2′
B3′
B4′
B5′
B6′
C2′
C3′
C4′
C5′
C6′
D3′
D4′
D5′
D6′

3.2 Columnar gene expression in specific neuroblasts of *Tribolium*

Columnar genes specify neuroblast fate along the DV axis in arthropods (Doeffinger and Stollewerk 2010, Wheeler et al 2005, reviewed in Skeath 1999). Wheeler *et al.* (2005) previously described the expression of columnar genes, and the interactions between them, in the ventral neuroectoderm of *Tribolium*. The authors did not, however, investigate the expression patterns in relation to single neuroblasts. To provide a basis for further analyses of the role of columnar genes on neural identity, the following section aims to assign the expression of the *Tribolium* columnar genes - *Tc-vnd, Tc-ind* and *Tc-msh* - to specific neuroblasts using the established neuroblast map (see Chapter 3.1).

Tribolium ventral nervous system defective is expressed in ventral column neuroblasts

Tc-vnd expression begins in the blastoderm in a longitudinal stripe on either side of the ventral midline (Wheeler *et al.* 2005). After the onset of gastrulation, the two stripes merge at the ventral midline (Fig. 3-2.1 A). Concurrently, anterior cells in the segment cease the expression of *Tc-vnd*, leaving a circle of *Tc-vnd* expressing ectodermal cells spanning the midline in the posterior part of each segment (Fig. 3-2.1 G). At this stage (NS3) no neuroblasts have delaminated yet. *Tc-vnd* expression extends along the AP axis, into the anterior part of a given segment and the anterior part of the segment posterior to it, coinciding with elongation of the germ band (Fig. 3-2.1 H). Midline cells still express *Tc-vnd* at this stage. *Tc-vnd* expression spans the whole medial column of ectodermal cells along a segment, except for ectodermal cells in the anterior part of a segment, corresponding to the area out of which neuroblasts of row two delaminate (Fig. 3-2.1 H). Around stage NS6 *Tc-vnd* is expressed in MP1, MP2, NBs 4-1, 5-2, 6-2 and 7-1 (Fig. 3-2.1 K). Although *Tc-vnd* is expressed in neuroectodermal cells out of which NB 1-1 arises (Fig. 3-2.1 H) no expression is detected in NB 1-1 after its delamination (Fig. 3-2.1 I). By stage NS8 expression of *Tc-vnd* in the midline has mostly ceased (Fig. 3-2.1 B, I). The number of *Tc-vnd* expressing neuroblasts increases with the formation of the *Tc-vnd* positive NBs 5-1, 6-1, 3-1 and 2-1 (Fig 3-2.1 I, L). Furthermore, expression of *Tc-vnd* ind neurons was detected but these were not analysed further in the current work (Fig. 3-2.1 E). It appears that neuroblasts 2-1 and 7-2 may express *Tc-vnd* from stage NS12

onwards (Fig 3-2.1 J). However, alterations in the neuroblast arrangement, caused by morphological changes of the neuromeres, prevent the assignment of *Tc-vnd* expression to specific neuroblasts. Consequently conclusive verification of *Tc-vnd* expression in NBs 2-1 and 7-2 is not possible. The schematic neuroblast map (Fig 3-2.1 L) depicts all neuroblasts which consistently express *Tc-vnd* (pink) and NBs 2-2 and 7-2 which are likely to express *Tc-vnd* (light pink). *Tc-vnd* expression decreases around stage NS14, with no expression in thoracic segments detectable at stage NS15 (Fig. 3-2.1 E, F).

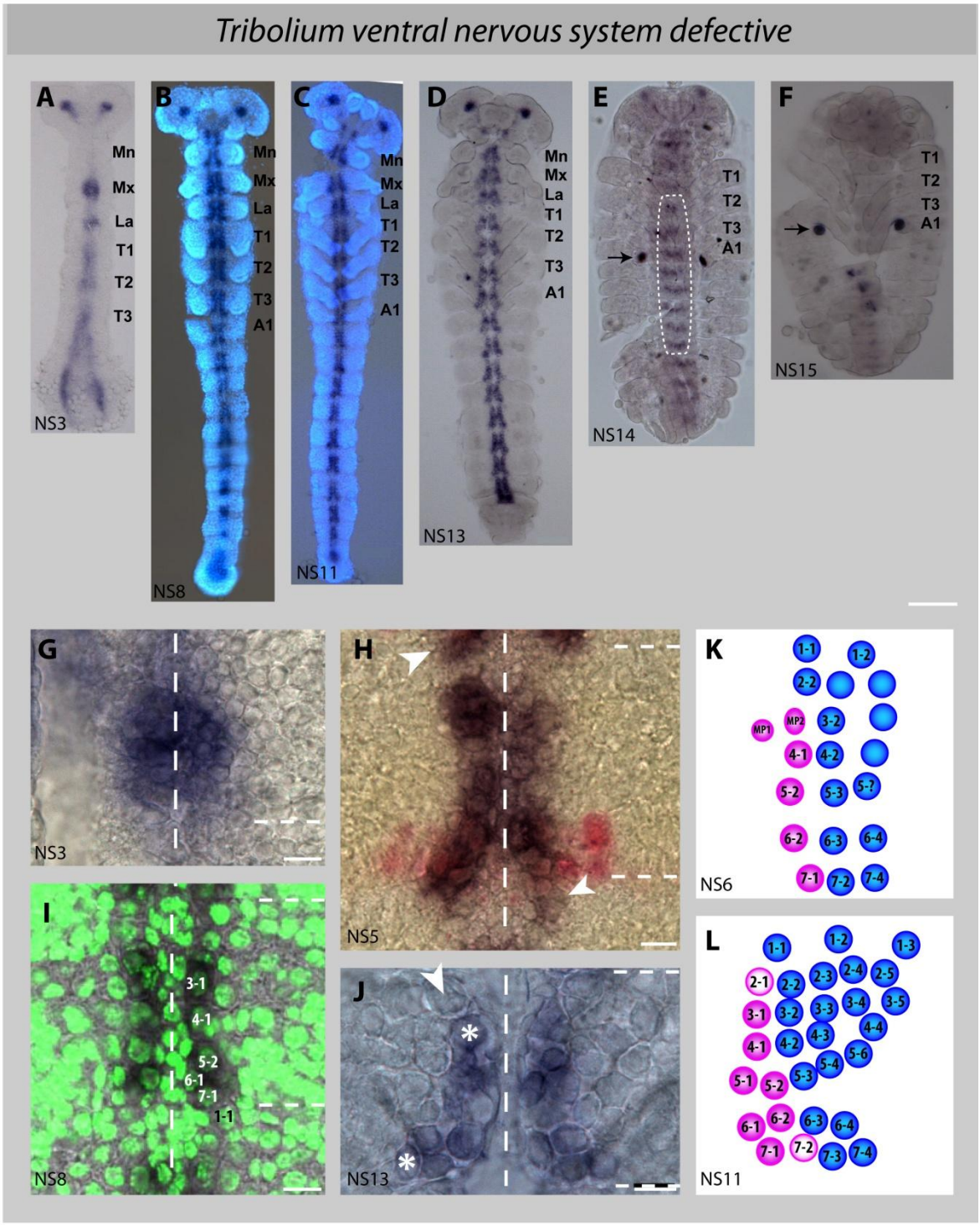


Figure 3-2.1: ***Tribolium vnd*** **expression in medial column neuroectoderm and neuroblasts**

Figure 3-2.1: *Tribolium vnd* expression in medial column neuroectoderm and neuroblasts.

Tc-vnd expression detected in embryos by *in situ* hybridisation (A to J). (A-F) *Tc-vnd* expression in successive developmental stages of *Tribolium*. (G-H) *Tc-vnd* expression in the first thoracic segment only. Anterior is towards the top. (E, F) Note background staining in pleuropodia (arrow). (E) Expression of *Tc-vnd* in neurons of thoracic and abdominal neuromeres (dashed oblong). (F) Expression of *Tc-vnd* in thoracic neuromeres has ceased, though there is still expression in abdominal neuromeres (G) At stage NS3 *Tc-vnd* expression becomes restricted to a cluster of ectodermal cells in the posterior part of a segment, spanning the midline. (H) Expression broadens along the AP axis, with no expression in a two to three cell wide row in the anterior part of a segment. *Tc*-En (red) is used as a marker for row six and seven and therefore verifies that *Tc-vnd* is expressed in ectodermal cells of the future row one neuroblasts (arrowhead) but not in row two. (I) Imaris section mode. A combination of bright field (*Tc-vnd* expression in black) and fluorescent microscopy (nuclei counter stain in green) was used to visualise the expression of *Tc-vnd* in NBs 3-1, 4-1, 5-2, 6-1 and 7-1 at stage NS8. (J) *Tc-vnd* expression in the first thoracic segment of the embryo shown in D. *Tc-vnd* is still expressed in neuroblasts along the ventral midline, with no expression in the most anterior neuroblast (arrowhead). Note that the neuroblast most anterior may be NB 2-1 (asterisk) and the most lateral posterior neuroblast may be NB 7-2 (asterisk). (K, L) Schematic neuroblast maps depicting *Tc-vnd* expression in ventral neuroblasts (pink) at stage NS6 (K) and NS11 (L). Possible expression of *Tc-vnd* in NBs 2-1 and 7-2 is shown in light pink. For abbreviations see abbreviation index page 10; segment borders are indicated by horizontal dashed lines; the midline is indicated by a vertical dashed line. scale bar = 100 µm (A-F); 10 µm (G-J).

Tribolium intermediate neuroblast defective is expressed in five intermediate column neuroblasts

Tc-ind expression in *Tribolium* begins some time after gastrulation in clusters of ectodermal cells situated in the intermediate part of the neuroectoderm, lateral to the *Tc-vnd* expression domain (Fig. 3-2.2 A, E). *Tc-ind* was not detected in the anterior part of any segment, corresponding to the neuroectoderm region out of which row one and two neuroblasts delaminate (Fig. 3-2.2 E, F). NS6 neuroblasts 3-2, 4-2, 5-3 and 6-3, all of which arise in the *Tc-ind* expressing domain, maintain *Tc-ind* expression after delamination (Fig. 3-2.2 F). Ectodermal cells cease *Tc-ind* expression concurrently with the delamination of *Tc-ind* expressing neuroblasts. Neuroblast 4-2 varies in its intensity of expression and appears to express *Tc-ind* only for a very limited period of time (Fig. 3-2.2 F (compare left and right hemineuromere)). Around stage NS7 NBs 3-2 and 4-2 cease the expression of *Tc-ind* altogether, leaving only NBs 5-3 and 6-3 expressing *Tc-ind* (Fig. 3-2.2 G). However, expression of *Tc-ind* in NB 5-3 is much weaker than initially (Fig. 3-2.2 G) In older embryos *Tc-ind* expression in NB 5-3 increases again but is still weaker than the expression in NB 6-3 at the same stage. Furthermore, the NS11 neuroblast 7-3 delaminates inside the embryo (Fig. 3-2.2 H). Expression is already detectable whilst NB 7-3 is still located in the ectoderm. Additionally, neuronal progeny developing out of *Tc-ind* positive neuroblasts express *Tc-ind* (Fig. 3-2.2 H', H''). At around stage NS11 a neuroblast in the medial row, possibly a row three neuroblast begins expressing *Tc-ind* (Fig. 3-2.2 H). As a consequence of cell movement in the hemineuromere it is not possible to assign this neuroblast with a definite identity. At stage NS14 one neuroblast at the lateral posterior edge of the segment maintains *Tc-ind* expression (Fig. 3-2.2 D).

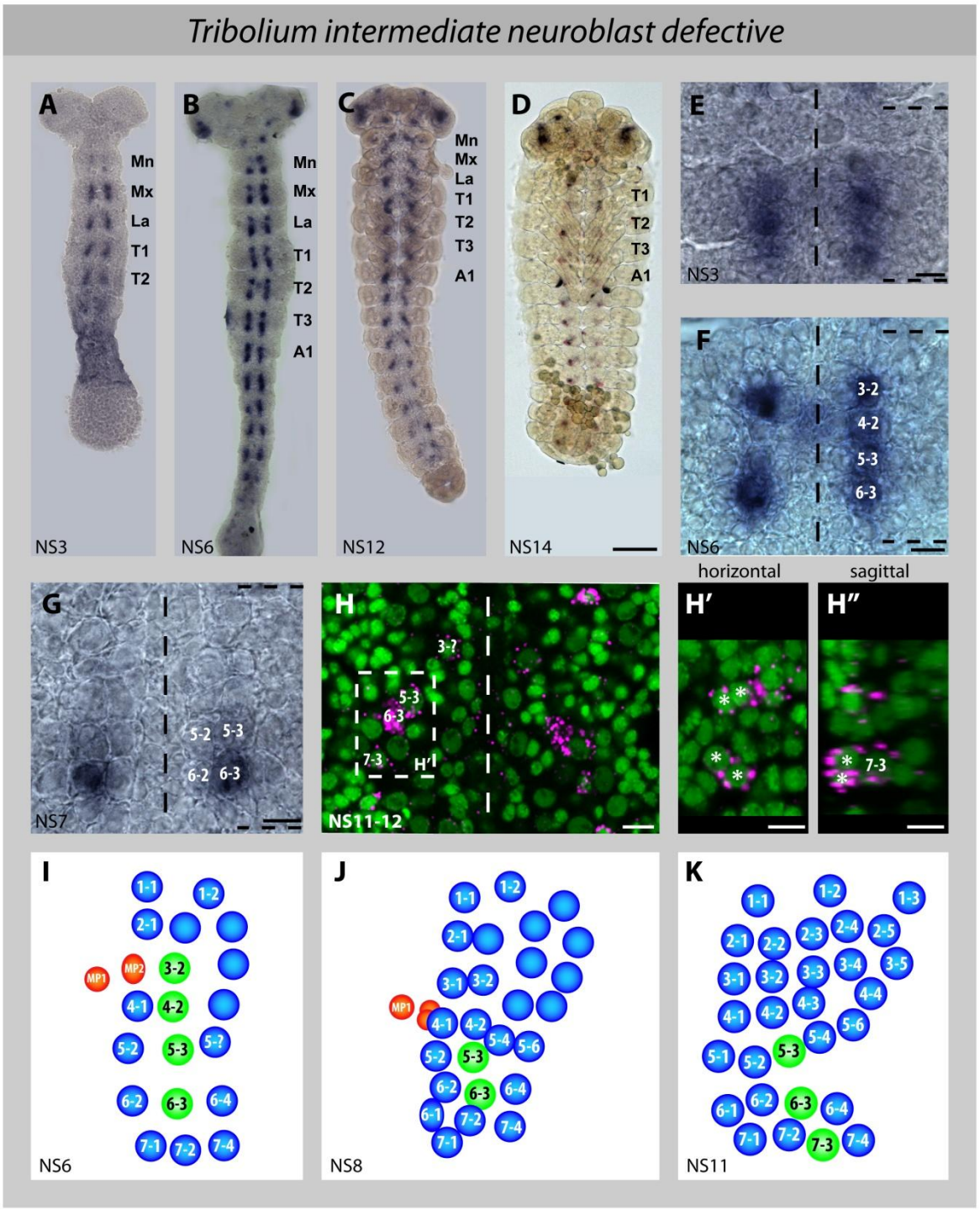


Figure 3-2.2: *Tribolium ind* expression in intermediate column neuroectoderm and neuroblasts.

Figure 3-2.2: *Tribolium ind* expression in intermediate column neuroectoderm and neuroblasts

Tc-ind expression detected in embryos by *in situ* hybridisation (A to H). (A-D) Overview of *Tc-ind* expression in embryos of different developmental stages. Anterior is towards the top. (E) First thoracic segment of the embryo in A showing *Tc-ind* expression in ectodermal cells along the intermediate column of the neuroectoderm. (F) Second thoracic segment of embryo B. At stage NS6 *Tc-ind* expression is detectable in intermediate column neuroblasts 3-2, 4-2, 5-3 and 6-3. (G) At stage NS7 a strong expression in NB 6-3 and a very faint expression in NB 5-3 are detectable. (H) Imaris section mode. Fluorescent *in situ* hybridisation (magenta) and, nuclei staining (green) in the first thoracic segment of a stage NS11-12 embryo. *Tc-ind* expression is detected in NBs 5-3, 6-3 and 7-3. Additionally, a neuroblast in row three expresses *Tc-ind.* (H') Magnification of dashed white box in H. *Tc-ind* is expressed in progeny of NBs 6-3 and 7-3 respectively. (H'') Sagittal section of H'. NB 7-3 with its progeny (shown by asterisk) basal to it. (I-K) Schematic neuroblast maps of Stage NS6, 8 and 11 depicting *Tc-ind* expression in neuroblasts (green) at the respective stage. For abbreviations see abbreviation index page 10; segment borders are indicated by horizontal dashed lines; the midline is indicated by a vertical dashed line; scale bar = 100 µm (A-D); 10 µm (E-H'').

Tribolium muscle segment homeobox is expressed in column three and four neuroblasts

Tc-msh exhibits the most dynamic expression of the three columnar genes analysed. *Tc-msh* expression in the thoracic segments is detected immediately after their formation in one large cluster of ectodermal cells (Fig. 3-2.3 A). The medial part of the cluster broadens along the AP axis. (Fig. 3-2.3 B, B'). As the germ band elongates expression in the thoracic segments extends to a two to three cell wide band of ectodermal cells expressing *Tc-msh* in different degrees of intensity along the lateral part of the neuroectoderm (Fig. 3-2.3 C, C'). The first identifiable neuroblast expressing *Tc-msh* is NB 6-4. Furthermore, a neuroblast in row two and the most lateral neuroblast of row four also express *Tc-msh*, but can not be assigned a specific neuroblast identity at that stage (Fig. 3-2.3 C', C''). With continuing development NBs 4-2, 4-3, 5-3 and 7-4 show *Tc-msh* expression. Furthermore, two neuroblasts in row two and three, neighbouring NBs 2-1 and 3-1 respectively, express *Tc-msh* (Fig. 3-2.3 G). Uncertainty regarding the pattern of neuroblast delamination in these two rows does not permit the neuroblasts to be assigned specific neuroblast identities. They may potentially be any of four neuroblasts: 2-2, 2-3, 3-2 or 3-3. At stage NS11 NBs 2-3, 3-3, 4-2, 4-3, 5-3, 5-4, 6-4 and 7-4 express *Tc-msh* (Fig. 3-2.3 E', E'', E'''). Lateral neuroblasts in row two to four do not express *Tc-msh.* Additionally at stage NS15 expression in neurons in the thoracic segments is detectable (Fig. 3-2.3 G).

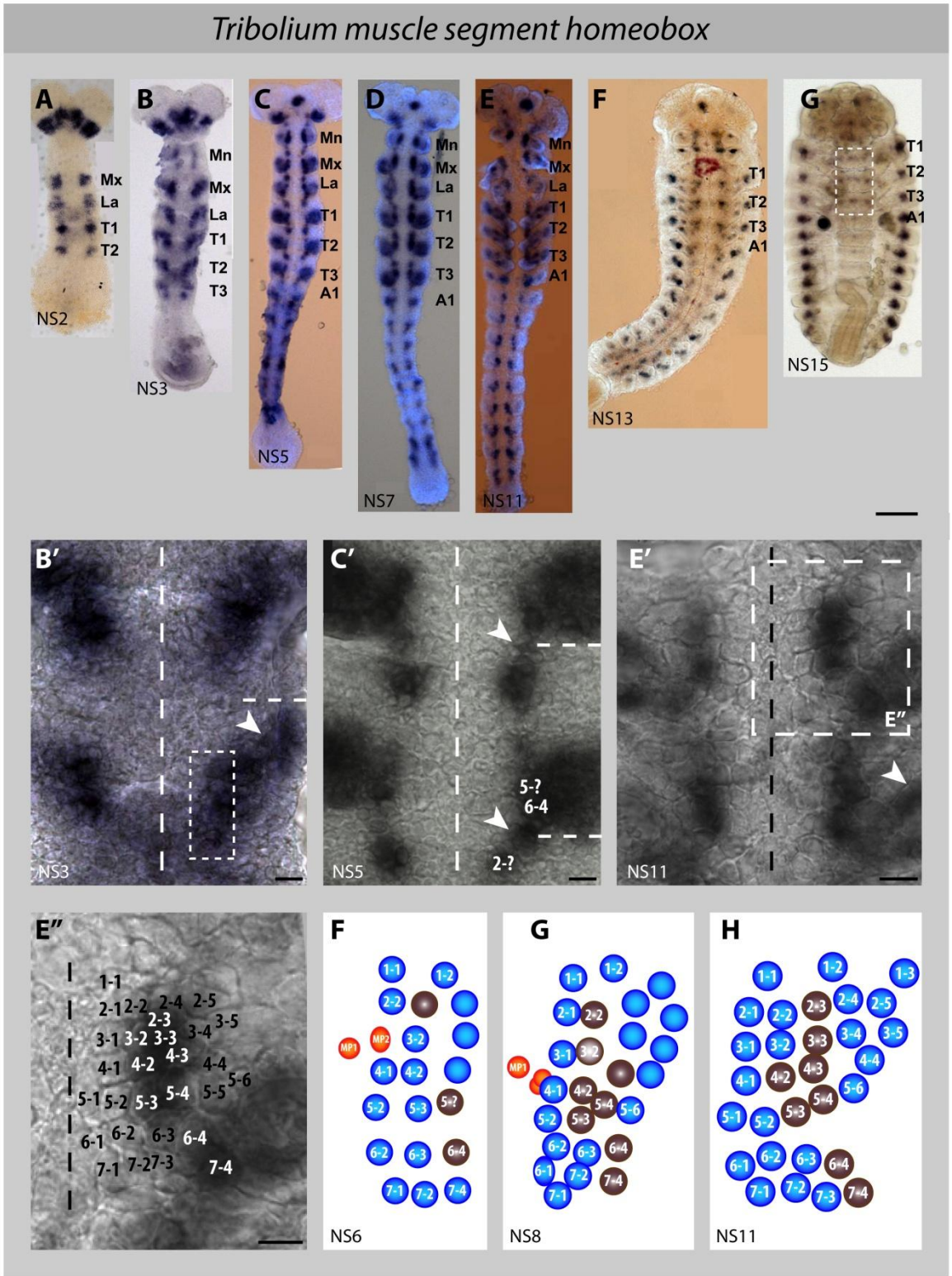


Figure 3-2.3: ***Tribolium msh*** **expression in the lateral neuroectoderm**

Figure 3-2.3: *Tribolium msh* expression in the lateral neuroectoderm

Tc-msh expression detected in embryos by *in situ* hybridisation (A-E''). (A-G) *Tc-msh* expression in successive developmental stages. Anterior is towards the top. (G) Note expression of *Tc-msh* in neurons of the thoracic segments (dashed box). (B') T1 and T2 of the embryo shown in B. *Tc-msh* is expressed in ectodermal cells of the future appendages (arrowhead) and the lateral neuroectoderm (dashed box). (C') T1 and T2 of the embryo shown in C. *Tc-msh* expression is detectable in a lateral row five neuroblast and NB 6-4. Note expression in ectodermal cells anterior to the row two neuroblast (arrowhead). (E') T1 and T2 of the embryo shown in E. *Tc-msh* is expressed in specific neuroblasts and the appendages (arrowhead). (E'') Magnification of dashed box in E' showing *Tc-msh* expression in neuroblasts of one hemineuromere. (F, G, H). Schematic neuroblast map of stage NS6, NS8 and NS11, respectively, with *Tc-msh* expression depicted in brown. For abbreviations see abbreviation index page 10; segment borders are indicated by horizontal dashed lines; the midline is indicated by a vertical dashed line; scale bar = 100 µm (A-G); 10 µm (B', C', E', E'').

3.3 Functional studies of columnar genes and their influence on neural subtype specific gene expression

The allocation of gene expression of the columnar genes *Tc-vnd, Tc-ind* and *Tc-msh* to specific neuroblasts in the previous result chapter enables to investigate their role in neural subtype specific gene expression. This ultimately permits conclusions to be drawn regarding their role in specifying neuronal identity. The current chapter is divided into two parts. Initially, overall phenotypes obtained by RNAi silencing of *Tc-ind*, *Tc-vnd*, *Tc-msh* are described. The second part analyses the function of *Tc-vnd* in neuron identity specification in detail, by comparing the expression of the neural subtype specific genes, *even-skipped* and *tailup*, in wild type embryos and *Tc-vnd*^{RNAi} embryos.

Tc-ind, *Tc-msh* and *Tc-vnd* RNAi

Injection of double stranded *Tc-msh* RNA does not cause any change to wild type expression of *Tc-msh*

The columnar gene *msh* is known to influence neural precursor identity in *Drosophila melanogaster* and the spider *Cupiennius salei.* Neuronal progeny of lateral neuroblasts rely on its expression in the respective neuroblasts/neural precursor groups for normal development (Isshiki *et al.* 1997, Döffinger and Stollewerk 2010). Four different double stranded (ds) RNA fragments, varying in size and target sequence (see Materials and Methods, appendix page 163), were injected into both pupae and embryos. However, no down regulation of *Tc-msh* was detected and in all cases *Tc-msh* expression in dsRNA injected embryos was indistinguishable from control embryos.

Tc-ind expression is down regulated in *Tc-ind*^{RNAi} embryos

Intermediate column neuroblasts (NBs 3-2, 4-2, 5-3, 6-3 and 7-3) and ectodermal cells express the columnar gene *Tc-ind* as described in the previous chapter (Fig. 3-3.1 A, B). The injection of dsRNA resulted in a decrease of *Tc-ind* expression (Fig. 3-3.1 C, D, E; see appendix for injection numbers). In most embryos a weak expression of *Tc-ind* in some intermediate column neuroblasts and ectodermal cells persisted (n=6 embryos; Fig. 3-3.1 C, D).

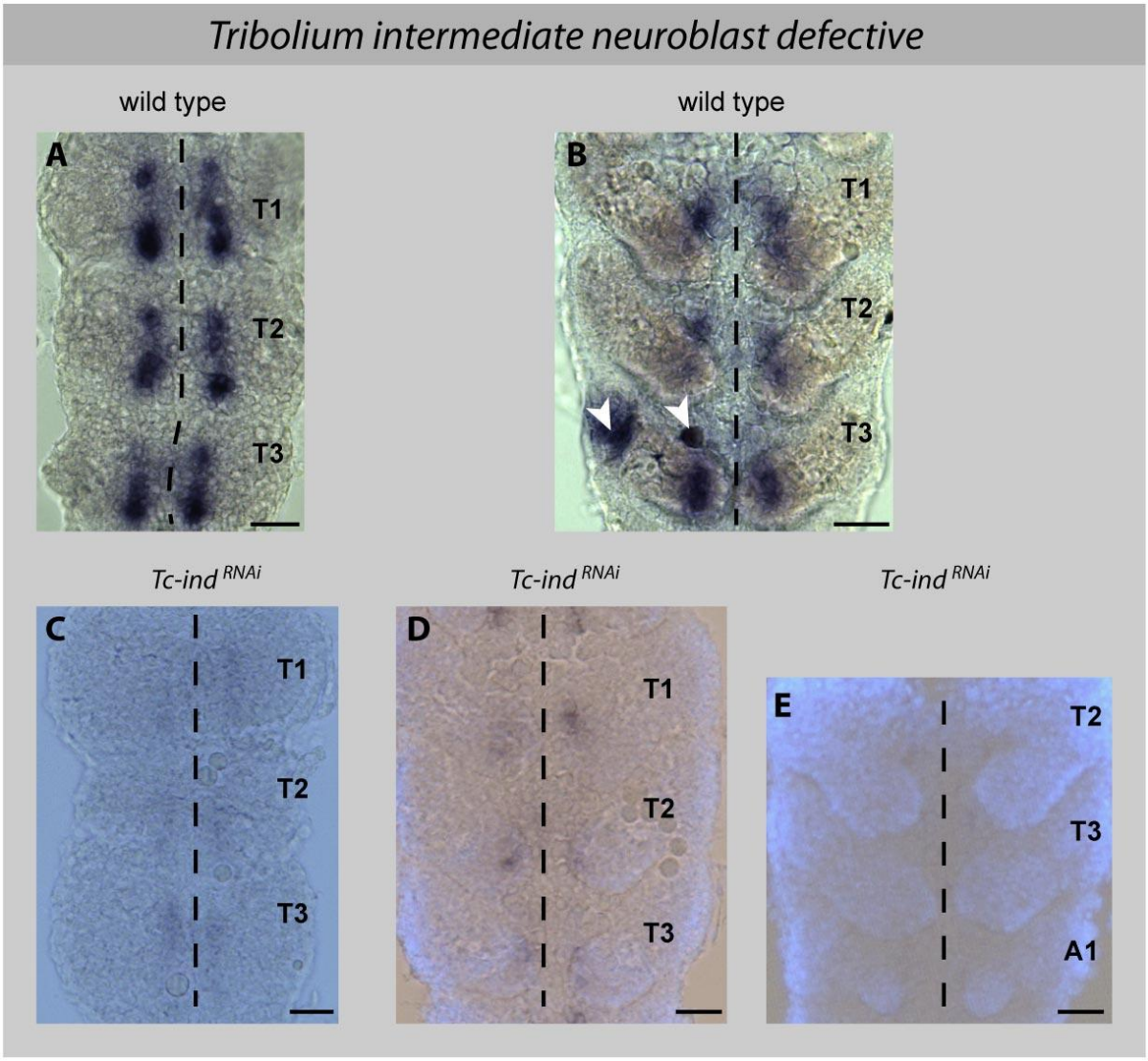


Figure 3-3.1: *Tribolium ind* expression in wild type and *Tc-ind*^{RNAi} embryos.

Tc-ind mRNA expression detected in the three thoracic segments of wild type and *Tc-ind*^{RNAi} embryos by *in situ* hybridisation. Anterior is towards the top. (A, B) *Tc-ind* is expressed in neuroblasts along the intermediate column of the ectoderm in wild type embryos. Note non-specific staining in the third thoracic segment of embryo in B (arrowhead). (C, D) *Tc-ind* expression is highly reduced in *Tc-ind*^{RNAi} embryos. (E) In some *Tc-ind*^{RNAi} embryos no *Tc-ind* expression is detectable. For abbreviations see abbreviation index page 10; the midline is indicated by a dashed line; scale bar = 25µm.

Silencing of *Tc-vnd* leads to loss of *Tc-vnd* expression and loss of neuroblasts

Tc-vnd is expressed in neuroblasts of column one and two in *Tribolium* (Fig. 3-3.2 A, E). Injection of ds *Tc-vnd* RNA resulted in loss of *Tc-vnd* expression and loss of neuroblasts of column one (Fig. 3-3.2 and Fig. 3-3.3). In the majority of embryos analysed there is no or very weak expression in the brain and neuroectoderm of gnathal and thoracic segments detectable (Fig. 3-3.2 B, D, G, H; see appendix for numbers of embryos analysed). Interestingly, many *Tc-vnd*^{RNAi} embryos exhibit *Tc-vnd* expression in the abdominal segments (Fig. 3-3.2 C). The strength of expression increases in abdominal segments formed later in development. At the time of injection the embryos have not formed any abdominal segments yet. Therefore, the expression of *Tc-vnd* in abdominal segments might be due to a decreasing effect of RNAi over time. However, since the *Tc-vnd* sequence used to synthesise dsRNA contains a highly specific homeobox, off-target effects are possible. To verify the results of the current work and exclude off-target effects a control experiment will have to be conducted. As a control at least one more double stranded *Tc-vnd* fragment, lacking the homeobox, will be injected and the results will be compared to the results obtained in the current work.

Loss of neuroblasts is verified by comparing the expression pattern of *Tc-ind* and *Tc-cas* in wild type and *Tc-vnd*^{RNAi} embryos (Fig. 3-3.3). In wild type embryos of stage NS5 *Tc-ind* is expressed in intermediate column neuroblasts separated from the midline by one column of neuroblasts (n=7 embryos; Fig. 3-3.3 A). In *Tc-vnd*^{RNAi} embryos *Tc-ind* expression expands into ectodermal cells of the medial column (data not shown, Wheeler *et al.* 2005). This expansion does not result in additional *Tc-ind* expressing neuroblasts. Instead it appears that medial neuroblasts 6-2, 5-2, 4-1 and the midline precursor cell MP2 are lost and as a result the intermediate *Tc-ind* expressing neuroblasts border the midline (Fig. 3-3.3 B). Expression of the temporal gene *Tc-cas* usually starts in neuroblasts lining the midline and a cell cluster out of which the MNB develops at stage NS8 (Fig 3.3-3 C). In *Tc-vnd*^{RNAi} embryos *Tc-cas* expression is altered with no expression in cells along the midline (n=4 embryos; Fig. 3-3.3 D).

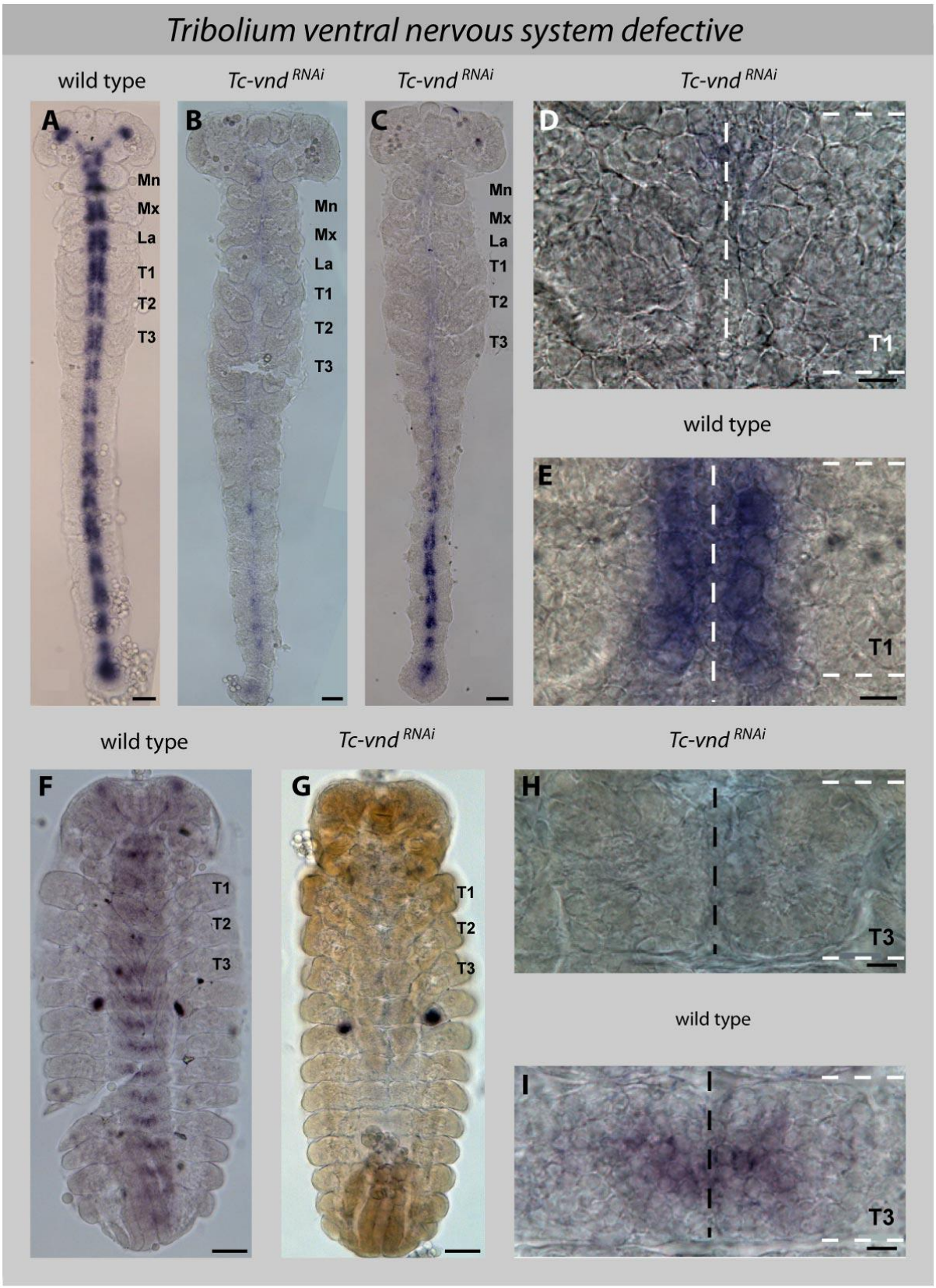


Figure 3-3.2: *Tribolium vnd* expression in wild type and *Tc-vnd*^{RNAi} embryos.

Figure 3-3.2: *Tribolium vnd* expression in wild type and *Tc-vnd*^{RNAi} embryos.

Tc-vnd expression in embryos (A, B, C, F, G) and the first (D, E) or third thoracic segment (H, I) of wild type and *Tc-vnd*^{RNAi} embryos detected by *in situ* hybridisation. Anterior is towards the top. (F, G) Dark staining in pleuropodia is non-specific staining. (A, E, F, I) *Tc-vnd* is expressed in ectodermal cells, neuroblasts and neurons along the ventral column of the hemineuromere in wild type embryos. (B, G) There is little or no *Tc-vnd* expression detectable in the head, gnathal and thoracic segments of *Tc-vnd*^{RNAi} embryos. (C) Note that in some *Tc-vnd*^{RNAi} embryos *Tc-vnd* expression is detected in abdominal segments. (D, H) In most *Tc-vnd*^{RNAi} embryos there is no expression of *Tc-vnd* detectable in T1/T3. For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line; segment borders are indicated by horizontal dashed lines; scale bar= 50µm (A, B, C, F, G); 10µm (D, E, H, I).

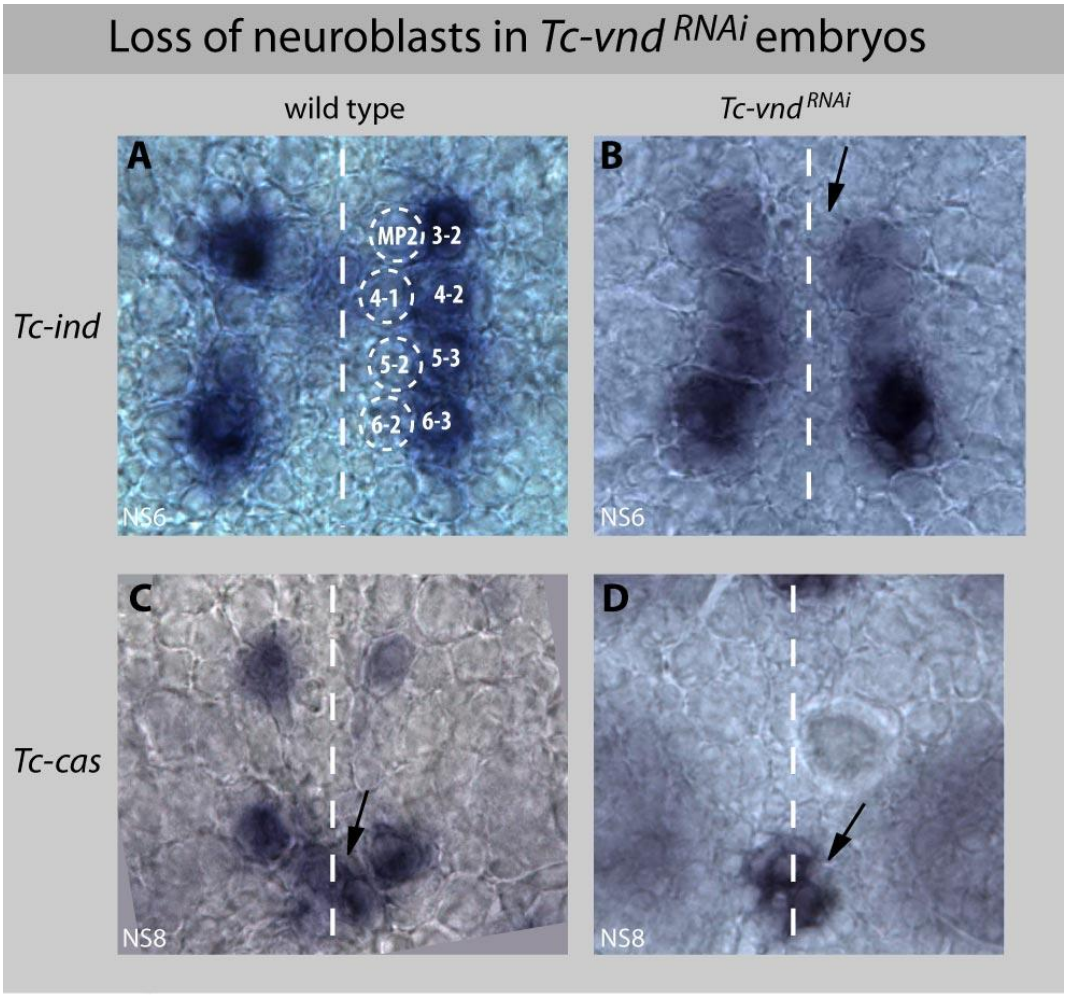


Figure 3-3.3: Ventral neuroblasts are lost in *Tc-vnd*^{RNAi} embryos.

Expression of *Tc-ind* and *Tc-cas* in T1 segments of wild type and *Tc-vnd*^{RNAi} embryos detected by *in situ* hybridisation (A-D). (A) In wild type embryos *Tc-ind* is expressed in intermediate column neuroblasts with a column of ventral neuroblasts medially (dashed white circles). (B) In *Tc-vnd*^{RNAi} embryos *Tc-ind* is expressed in neuroblasts along the midline, with no neuroblasts medial to them (arrow). (C) *Tc-cas* is expressed in neuroblasts along the ventral midline and cells in the region of the future MNB (arrow) in stage NS8 wild type embryos. (D) In stage NS8 *Tc-vnd*^{RNAi} embryos *Tc-cas* is only expressed in the cluster of cells out of which the MNB delaminates (arrow). For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line.

Additional cluster of *Tc*-Eve neurons occurs in *Tribolium*

The pair rule gene *even-skipped* is expressed from an early stage onwards in *Tribolium*, operating as a segmentation gene (Brown *et al.* 1996). To date, there are no published data available on its expression in the nervous system. The antibody 3C10, originally produced against grasshopper Eve (Patel *et al.* 1992), also detects EVE protein in *Tribolium* (Brown *et al.* 1996). *Tc*-Eve expression in the nervous system begins with the formation of the first neurons around stage NS8 of development. Expression in neuroblasts was not detected at any time. The final number of *Tc*-Eve expressing cells is on average 22 cells per thoracic and first abdominal hemineuromere with the highest number counted 24 (n=18 hemineuromeres) (Fig. 3-3.4 H). Around stage NS8 two cells per thoracic hemineuromere, one in the anterior part, close to the preceding segment, and one in the middle express *Tc*-Eve (Fig. 3-3.4 A). In further developed segments there are respectively two cells expressing *Tc*-Eve in the position of the former one cell (Fig 3-3.4 A). The positions of these cells resemble the arrangement of the Eve^+ RP2, RP2 sibling, aCC and pCC neurons in *Drosophila* (Broadus *et al.* 1995). In *Drosophila* the RP2 and its sibling neuron are formed by GMC 4-2a with the RP2 sibling neuron only transiently expressing Eve (Broadus *et al.* 1995). And indeed also in *Tribolium* the expression decreases in one of the two cells shortly after their formation and eventually ceases (Fig. 3-3.4 B). aCC and pCC are generated by the first GMC 1-1a, the first GMC of NB 1-1 in *Drosophila* and are characterised by their migrating into the anterior lying segment, eventually becoming situated next to the anterior commissure (Broadus *et al.* 1995). Also in *Tribolium* the two neurons, formed by the more anterior lying cell of the originally two cells per segment, migrate towards the posterior part of the anterior lying immediately after their formation segment (Fig. 3-3.4 A). Due to the position and the migration process these cells are likely to be homologous to the aCC and pCC neurons in *Drosophila.* Meanwhile, there are more cells formed basally to the aCC and pCC neurons (Fig. 3-3.4 B). According to their position they resemble the U/CQ neurons of *Drosophila*. In the oldest stage analysed 5 U/CQ neurons basal to the aCC/pCC neuron express *Tc*-Eve (n=28 hemineuromeres) (Fig. 3-3.4 D). When around two to three U/CQ neurons have formed, neurons in the lateral anterior part of the hemineuromere start expressing *Tc*-Eve (Fig. 3-3.4 C). They correspond to the *eve*-expressing lateral neurons (EL cells) in *Drosophila*. In the oldest embryos up to 13 EL cells were counted with an average of 10

EL cells (n=18 hemineuromeres). Eventually, cells in the posterior lateral part of the hemineuromere, lateral to the U/CQ cells, begin expressing *Tc*-Eve around stage NS14 (Fig. 3-3.4 D). This cell cluster, consisting of up to 7 cells in the oldest analysed stage, has not been observed in *Drosophila* and is termed, on the basis of its position, the posterior Eve lateral cluster (PEL).

Knockdown of *Tc-vnd* function causes failure of neuron formation and changes in migration patterns

In *Tc-vnd*^{RNAi} embryos *Tc*-Eve expression is altered in various ways. These alterations, however, do not only occur between different embryos but also between different segments of the same embryos. Of 14 embryos analysed, one exhibited no phenotype. All embryos exhibiting a phenotype developed RP2 and EL neurons as seen in wild type (Fig. 3-3.4 E, F). Changes occur in the formation of the aCC/pCC and U/CQ neurons. In 61% of hemineuromeres aCC and pCC are formed (Fig. 3-3.4 F), in 9% none and in 30% only one cell, which could be either aCC or pCC or the GMC, is formed (n=144 hemineuromeres; Fig. 3-3.4 E). Additionally, their usual migration pattern can be affected. In wild type embryos aCC and pCC neurons migrate from the anterior part of a segment into the posterior part of the adjacent segment to find their final place close to the posterior commissure (Fig. 3-3.4 G). In 32% of hemineuromeres of *Tc-vnd*^{RNAi} embryos aCC and pCC neurons do not migrate (Fig. 3-3.4 E). U/CQ neurons are not formed in any hemineuromeres of *Tc-vnd*^{RNAi} embryo analysed except for three (2%) (Fig. 3-3.4 E, F). PEL neurons are detected in *Tc-vnd*^{RNAi} embryos from stage NS14 onwards (Fig. 3-3.4 F) as seen in wild type (Fig. 3-3.4 G).

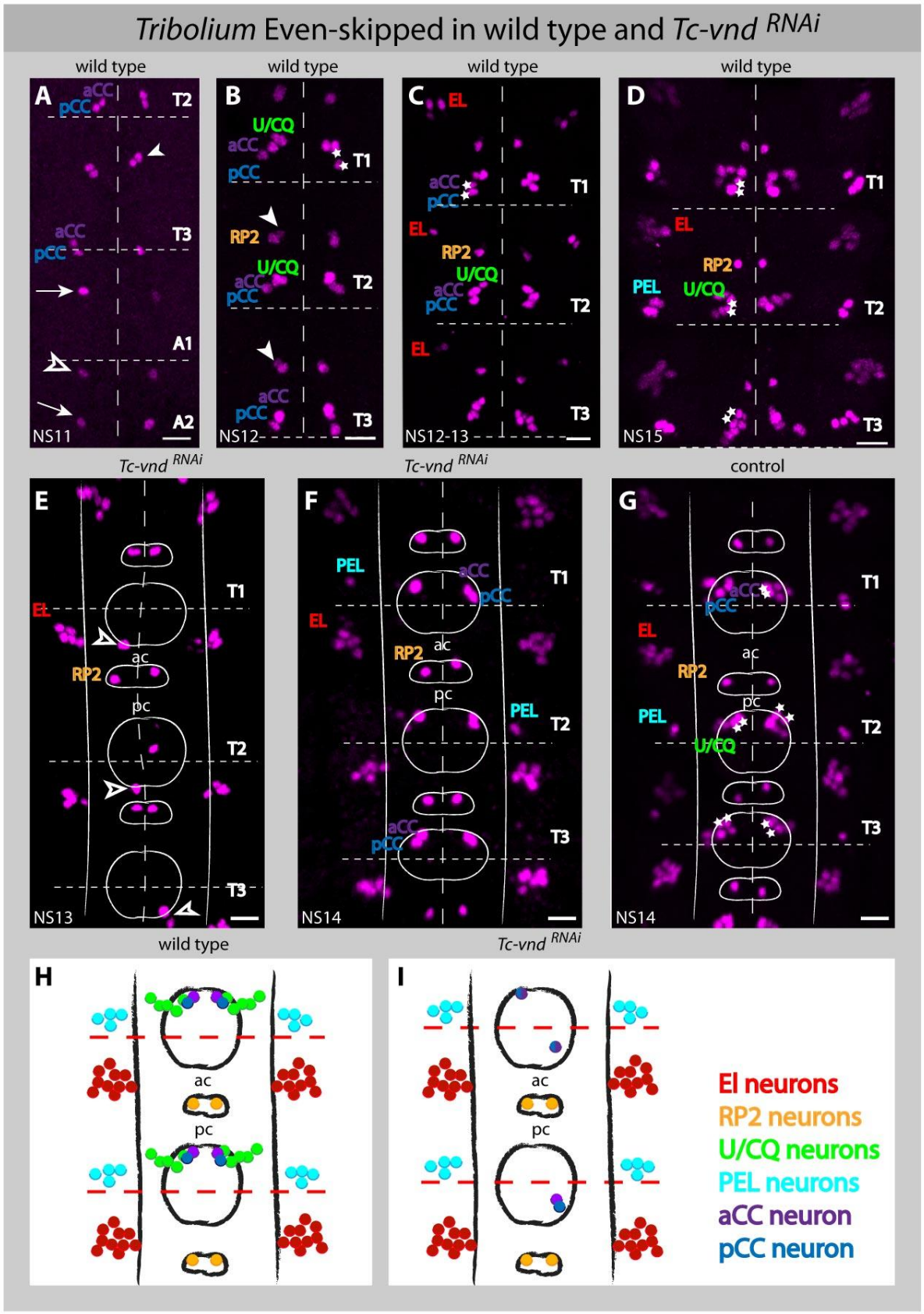


Figure 3-3.4: *Tc*-Eve expression in wild type and *Tc-vnd*^{RNAi} embryos

Figure 3-3.4: *Tc*-Eve expression in wild type and *Tc-vnd*^{RNAi} embryos

Tc-Eve expression detected by the α-Eve antibody (3C10). Imaris volume mode (A-G); T1, T2 and T3 segments except for A; A-D show the formation of *Tc*-Eve expressing neurons in successive developmental stages in wild type embryos. Asterisks indicate aCC/pCC neurons (B, C, D, G). (A) Note that the second and third thoracic segments, plus abdominal segments A1 and A2, of a stage NS11 embryo are presented. Both neuroblasts and neurons in consecutive segments exhibit temporal asynchrony in their formation, with those in a given segment exhibiting slightly more advanced development than those in the segment posterior to it. Therefore the expression pattern of *Tc*-Eve in the first two abdominal segments of a stage NS11 embryo is consistent with the expression pattern in the thoracic segments of a stage NS8 embryo. The first *Tc*-Eve^{+} cells in each hemineuromere are likely to be homologues of GMC 4-2a (arrow) and GMC 1-1a (open arrowhead) in *Drosophila*. Subsequently, GMC 4.2a divides forming the *Tc*-Eve^{+} RP2 neuron and the RP2 sibling cell, which transiently expresses *Tc*-Eve (T3, arrowhead). Additionally, GMC 1-1a divides, generating aCC and pCC. Note that aCC and pCC migrate over the segment border into the posterior part of the preceding segment (T3 and T2). (B) The next *Tc*-Eve^{+} neurons to be formed are the U/CQ neurons (T2). Note that *Tc*-Eve expression in the RP2 sibling decreases (arrowheads) before ceasing eventually (T1). (C) Shortly after, the EL neurons are formed. (D, G) From stage NS 14 onwards *Tc*-Eve^{+} neurons in the lateral posterior part of a hemineuromere are formed (PEL cluster). (E, F, G) The axonal scaffold is indicated by the solid white lines. (E, F) *Tc*-Eve expression in *Tc-vnd*^{RNAi} embryos. EL neurons and RP2 neurons are formed as in wild type. Note that aCC and pCC neurons are formed in some neuromeres (F), but not all (E). (E) In some cases only aCC or pCC alone, or neither, is formed. Additionally the aCC and pCC neurons do not always migrate into the posterior part of the preceding segment (open arrowhead). (F) PEL neurons are formed as in wild type. (G) Wild type embryo at a similar stage as the *Tc-vnd*^{RNAi} embryo shown in F. (H) Schematic drawing of *Tc*-Eve^{+} neurons depicting the average number of *Tc*-Eve^{+} cells in a wild type embryo. (I) Illustration of *Tc*-Eve^{+} neurons in a *Tc-vnd*^{RNAi} embryo. Note that single cells shown in dual-colours (violet and blue) indicate uncertainty about cell identity (*i.e.* they could be aCC, pCC or the precursor GMC). The horizontal red dashed lines indicate the segment borders. The black lines indicate the axonal scaffold; anterior commissure (ac); posterior commissure (pc); the midline is indicated by the vertical dashed line; the segment borders by the dashed horizontal lines; scale bar = 10µm.

Table 3-3.1: *Tc*-Eve expression in *Tc-vnd*^{RNAi} *embryos*

The second column in the table provides the total number of hemisegments counted with percentage in parentheses. The third column provides the number of hemisegments where aberrant migration of neurons occurred. The percentage in parentheses refers to the number of hemisegments where the respective phenotype occurs. Not applicable (NA).

Phenotype of *Tc*-Eve expression in *Tc-vnd*^{RNAi} embryos	Number of hemisegments	Aberrant migration number of hemisegments
Total	144 (100%)	32 (24%)
aCC/pCC	88 (61%)	13 (14.7%)
aCC, pCC or GMC	43 (30%)	19 (44%)
neither aCC nor pCC	13 (9%)	NA
U/CQ	3 (2%)	NA

The neural subtype specific gene *tailup* is expressed in up to 57 neurons per hemineuromere in *Tribolium*

The expression pattern of the neural differentiation marker *Tc-tup* in *Tribolium* was analysed via *in situ* hybridisation and antibody staining. As the 40.3A4 anti-rat islet1 antibody also detects Tup expression in *Drosophila*, cross-reactivity in *Tribolium* was evaluated. Comparison of *in situ* hybridisation and antibody staining reveals that the 40.3A4 islet1 antibody against rat specifically detects Tup in *Tribolium* (Fig. 3-3.5 I and K (mRNA), J (protein)). In early stages of Tribolium *Tc*-Tup is expressed in the labrum, stomodeum, lateral protocerebrum and the growth zone (Fig. 3-3.5 A, B). At stage NS3 expression in the future heart commences, followed by expression in the proctodeum (Fig. 3-3.5 B, C, D). Expression in the nervous system is restricted to post mitotic neural cells and can be detected from stage NS12 onwards (Fig. 3-3.5 E). Similar to the anterio-posterior gradient of neuroblast formation between the three thoracic segments a gradient of neuron formation is observed (Fig. 3-3.5 E, H). The first neurons expressing *Tc*-Tup lie in the anterior middle of a neuromere (Fig. 3-3.5 H; Fig. 3-3.7 A). Expression expands medially and to the posterior of the neuromere (Fig. 3-3.5 H (T1); Fig. 3-3.7 B, C). Other than a few neurons in the posterior part the majority of Tup expression is restricted to the anterior two thirds of the neuromere (Fig. 3-3.5 I, J, K). Eventually around 50 neurons are *Tc*-Tup^{+} per hemineuromere (n=20) with up to 57 neurons counted (Fig. 3-3.7 C). A single large, strongly expressing cell forms along the midline between the anterior and posterior commissure (Fig. 3-3.5 J; Fig. 3-3.6 C, C', C''). According to its position it is the homologue of the H-cell in *Drosophila* (Bossing and Technau 1994). Basally to it three to four neurons are expressing *Tc*-Tup (Fig. 3-3.6 C, C', C''; Fig. 3-3.7 C). Three of them correspond to the *tup*$^{+}$ RP1, 3, 4 neurons in *Drosophila* (Thor and Thomas 1997). Additionally, there are two more conspicuous neurons formed apically to it (Fig. 3-3.6 C', C''; Fig. 3-3.7 C).

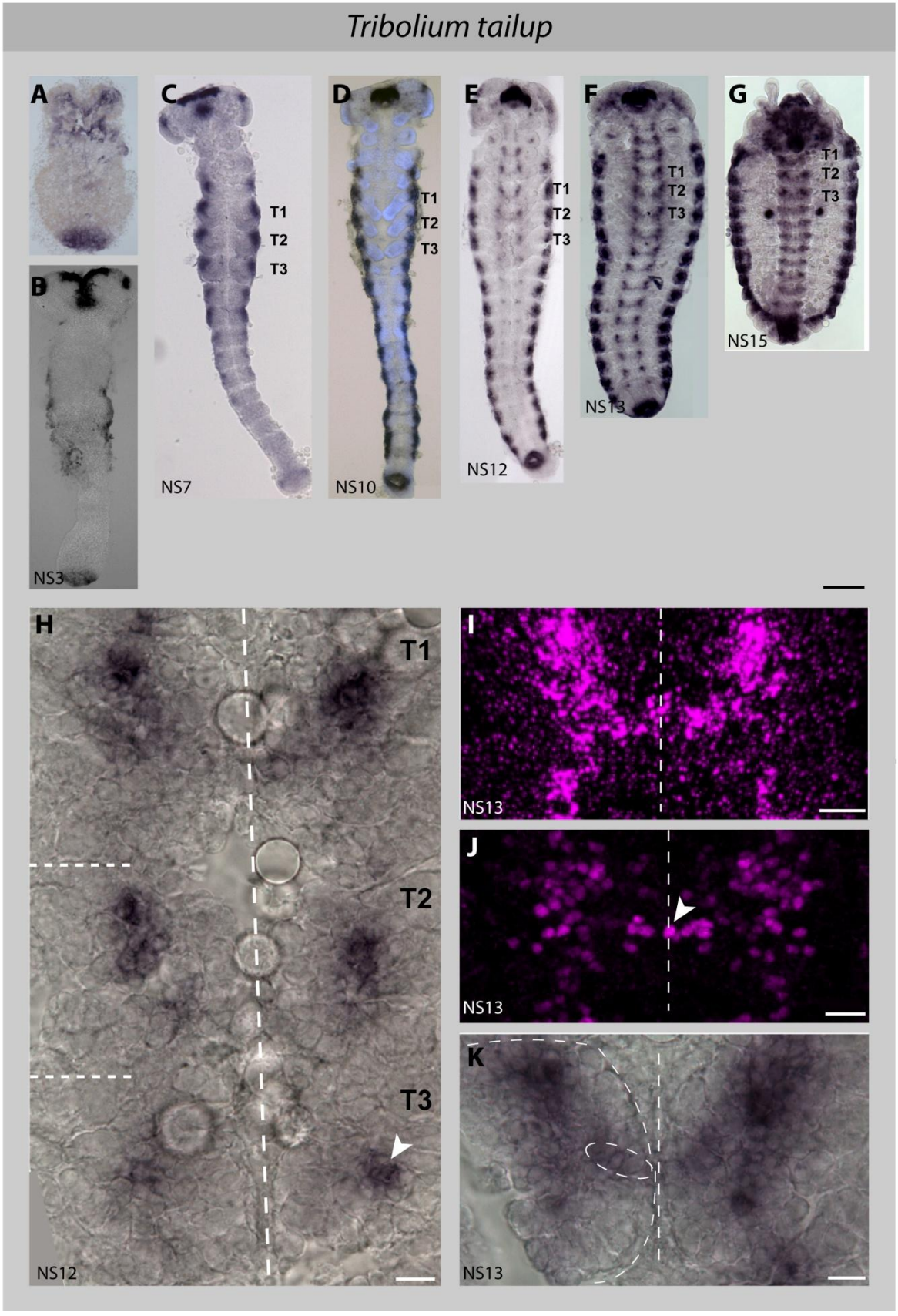


Figure 3-3.5: *Tribolium tailup* mRNA and Tailup protein are expressed in a similar pattern.

Figure 3-3.5: *Tribolium tailup* mRNA and Tailup protein are expressed in a similar pattern.

Tc-tup expression in embryos (A-G), in the three thoracic segments of a stage NS12 embryo (H) and the first thoracic segment of a stage NS13 embryo (I, J, K) detected by chromogenic (A-H, K) or, fluorescent (I) *in situ* hybridisation and antibody staining against *Tc*-Tup protein (J). (A, B, C, D) *Tc-tup* is expressed in the head, future heart tissue, growth zone and the proctodeum. (E, F, G) *Tc-tup* expression in the nervous system is first detected at stage 12 (dashed box) and lasts until the last stage analysed (NS15). (H) *Tc-tup* expression in T1, T2, T3 of the embryo shown in E. Note the gradient of *Tc-tup* expression between T1 to T3. In T3 *Tc-tup* is expressed in two cells in the anterior lateral part of a hemineuromere (arrowhead). The expression broadens along the AP axis (T2, T1). (I, K) *Tc-tup* is expressed in neurons along the lateral part of a hemineuromere and neurons in the middle of a segment (dashed circle) in stage NS13 embryos. (J) *Tc*-Tup protein is expressed in a similar pattern as *Tc-tup* mRNA (compare with I, K). Note the large cell (H-cell) along the midline (arrowhead) For abbreviations see abbreviation index page 10; the midline is indicated by the vertical dashed line, the segment borders by the horizontal dashed lines; scale bar = 100 µm (A-G), 10 µm (H-K).

Knockdown of *Tc-vnd* function shows reduction of *Tc*-Tup expressing neurons

In *Tc-vnd*^{RNAi} embryos *Tc*-Tup expression is not disrupted in most neurons. Noticeable, however, is a loss of neurons between the anterior and posterior commissures and on the level of the posterior commissure, with only the H-cell remaining (Fig. 3-3.6 B, D). Analysis of transverse sections reveals that the number of RP neurons is reduced, as are numbers of the cells lying apical to the H-cell (Fig. 3-3.6 D', D''; Fig. 3-3.7 D).

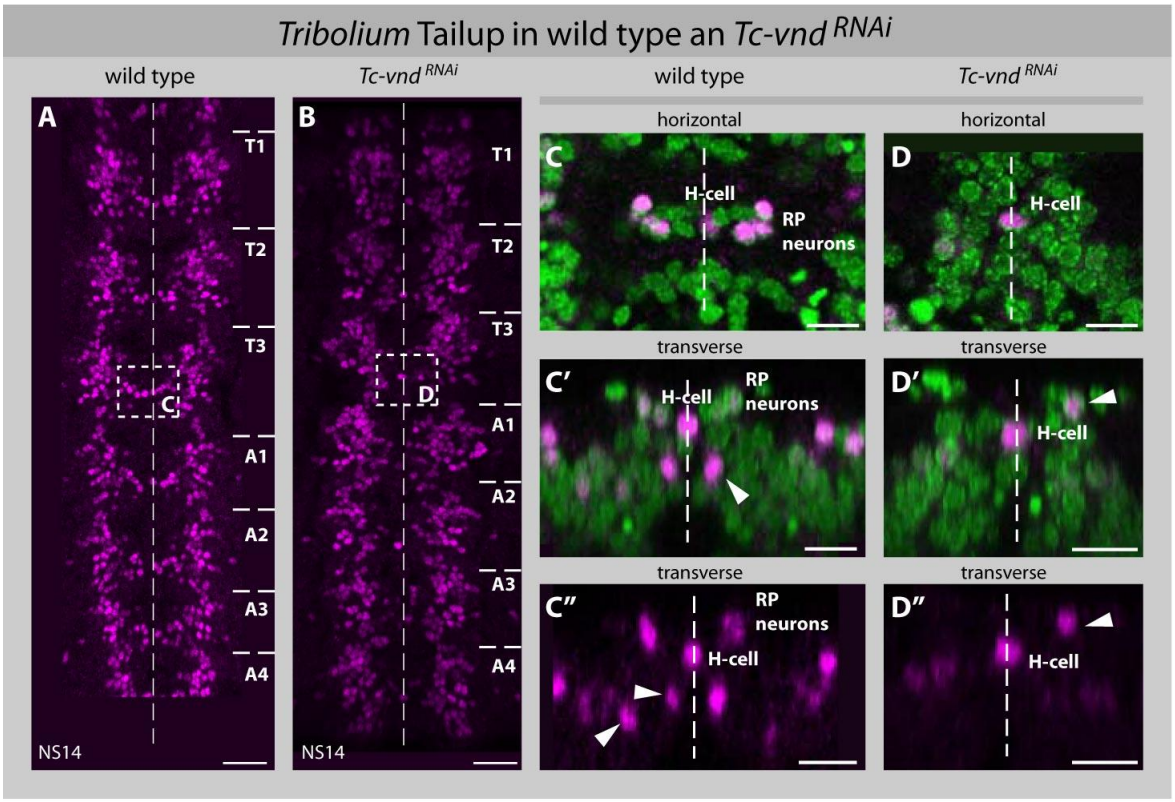


Figure 3-3.6: RP neurons are not formed in *Tc-vnd*^{RNAi} embryos

Tc-Tup expression in wild type and *Tc-vnd*^{RNAi} detected using the 40.3A4 antibody (magenta). Cell nuclei are visualised with the nuclei marker SYTOX® Green (green). Imaris volume mode, anterior is towards the top (A-B), horizontal sections (C, D), transverse sections (C', C'', D', D'') with basal towards the top. (A) Wild type expression of *Tc*-Tup in a stage NS14 embryo in segments T1-A4. (B) Loss of *Tc*-Tup expressing neurons in the middle of the hemineuromere (dashed box) in an embryo at the same stage as shown in A. (C) Horizontal section of similar position as in A (dashed box). *Tc*-Tup is expressed in the RP-neurons and H-cell in wild type. (C', C'') Transverse section of C. *Tc*-Tup is expressed in RP neurons basally, the H-cell in the middle and two cells apical to the H-cell (arrowhead). (D) Horizontal section of similar position as shown in B (dashed box). In *Tc-vnd*^{RNAi} embryos only the H-cell remains. (D', D'') Transverse section of D. Verification of loss of the RP neurons and neurons lying apical to the H-Cell in *Tc-vnd*^{RNAi} embryos. Note that there is one neuron basal to the H-cell expressing *Tc*-Tup (arrowhead). For abbreviations see abbreviation index page 10; the midline is indicated by a vertical dashed line; segment borders by horizontal dashed lines; scale bar = 20µm (A, B); 10µm (C, D).

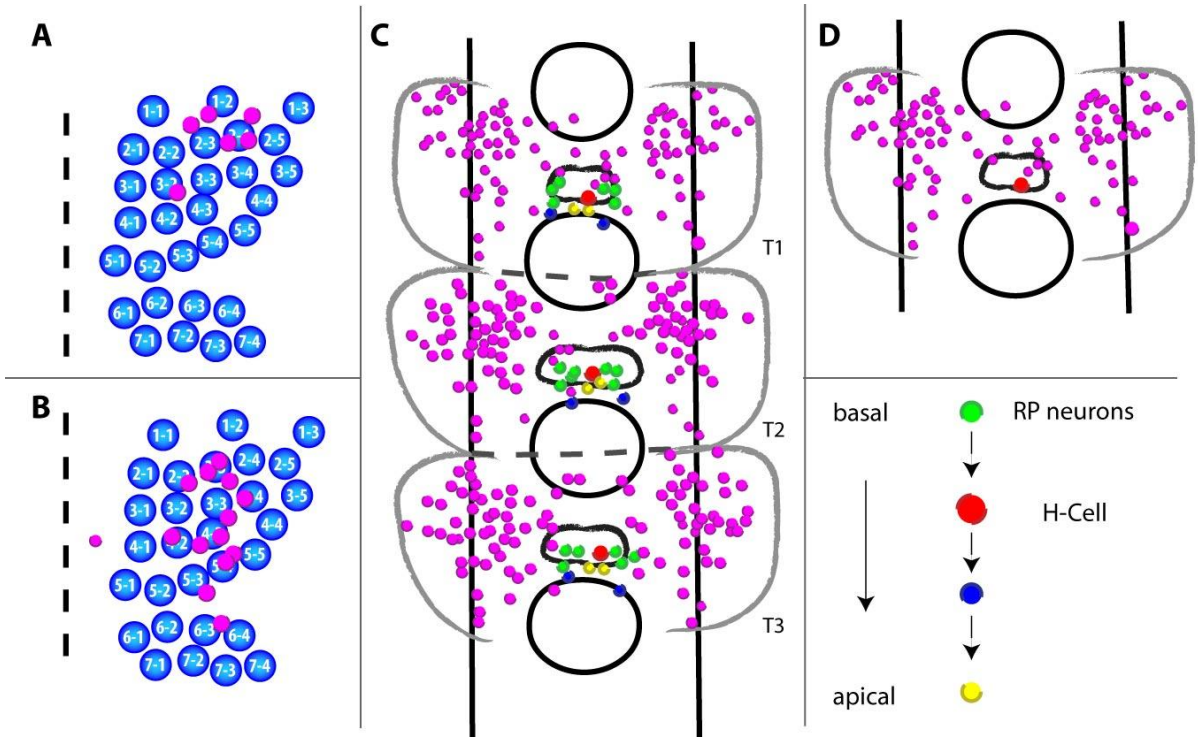


Figure 3-3.7: Schematic drawing of *Tc*-Tup expressing neurons in wild type and *Tc-vnd*^{RNAi} embryos.

(A, B) One thoracic hemineuromere with all thoracic neuroblasts shown, illustrating the area out of which *Tc*-Tup expressing neurons (pink) arise in wild type embryos at stage NS12 (A) and between NS12 and NS13 (B). (C) *Tc*-Tup^{+} neurons in the three thoracic segments (pink) of wild type embryos. The different coloured neurons can be uniquely identified by their position along the apico-basal axis. The H-cell (shown in red) is formed along the midline, three to four RP neurons (green) are positioned basally, whilst apically two more *Tc*-Tup^{+} neurons are formed (blue and yellow). (D) In *Tc-vnd*^{RNAi} embryos the RP neurons and the two neurons apical to the H-cell are not formed. The axonal scaffold is shown in black, the midline is indicated by the vertical dashed line and the neuromere outline is indicated in grey.

Silencing *Tc-ind* did not show any changes in the expression pattern of *Tc*-Eve and *Tc*-Tup

Knock down of *Tc-ind* did not produce any detectable changes in the expression of *Tc*-Eve and *Tc*-Tup. This, however, must be considered to be a preliminary finding, due to the limited number of embryos analysed (n=4). It was expected that in case the intermediate column neuroblasts are missing there should be no RP2 neurons visible, being a direct progeny of NB 4-2.

4 Discussion

The general pattern of neuroblast formation is conserved between insects

The comparison of early neurogenesis in all insect species analysed to date reveals that the number and arrangement of neuroblasts is highly conserved (Truman and Ball 1998: *Ctenolepisma longicaudata*; Bate 1976: *Locusta migratoria*; Doe and Goodman 1985: *Schistocerca americana*; Shepherd and Bate 1990: *Schistocerca gregaria*; Tamarelle *et al.* 1988: *Carausius morosus* and Hartenstein and Campos-Ortega 1984; Doe 1992: *Drosophila melanogaster*). The results obtained for *Tribolium* neuroblast formation largely resemble what is known for *Drosophila*. As in *Drosophila* neuroblasts delaminate and form a cell layer between mesoderm and ectoderm in *Tribolium*. During delamination the neuroblasts form a bottle shaped cell with the nucleus basal and the cell membrane attached apically to the ectoderm in both insects. Eventually the neuroblast loses contact with the ectoderm and forms a large round cell in a distinct layer between the ectoderm and mesoderm. As in all other insects analysed, 30 neuroblasts and one unpaired median neuroblast (MNB) delaminate in the six thoracic hemisegments, resulting in a pattern of seven rows with three to five neuroblasts each (Fig. 4-1.1 NS11).

In *Drosophila* neuroblasts delaminate in five temporally distinct segregation waves (S1-S5) (Fig. 4-1.1; Hartenstein and Campos-Ortega 1984, Doe 1992). Originally three waves were described (S1-S3) (Hartenstein and Campos-Ortega 1984). The first segregation wave (S1) is divided into two waves, early S1 and S1. During early S1 at late embryonic stage 8, neuroblasts delaminate in the medial and lateral column with additionally one neuroblast in the intermediate column. A further neuroblast is added during the early embryonic stage 9, completing the first segregation wave. The second wave is characterised by the delamination of mainly intermediate column neuroblasts. Hartenstein and Campos-Ortega (1984) described a further wave (S3) but envisaged problems analysing it on a morphological basis alone. Implementing molecular data Doe (1992) refined the later pattern of neuroblast formation by introducing a division of the third wave into two further waves (S4-S5).

In *Schistocerca* and *Ctenolepisma* neuroblasts delaminate continuously (Doe and Goodman 1985, Truman and Ball 1998). Nevertheless, neuroblasts are formed in a temporally stereotypic sequence. Therefore, Doe and Goodman (1985) distinguished between early, middle and late forming neuroblasts. Detailed studies analysing the exact time each individual neuroblast is formed, revealed that the first neuroblast is always NB 3-5 followed by NB 2-5. The next eight neuroblasts in *Schistocerca* delaminate almost simultaneously reflecting a pattern similar to that in *Drosophila* S1 neuroblasts. Subsequently neuroblasts form sequentially, with each neuroblast delaminating within a specific time span (Doe and Goodman 1985).

In *Tribolium* no temporally distinct waves of neuroblast delamination were observed. However, as in *Schistocerca* the sequence in which neuroblasts delaminate appears to be consistent. During the course of neurogenesis, different stages of neuroblast development are observable at every stage, with neuroblasts just commencing delamination, whilst others are in the process of delamination (forming bottle shaped cells) and others have already fully delaminated with no attachment to the ectoderm left. Furthermore, analysing the expression of the neuroblast marker *Tc-ase* also demonstrated a sequential pattern of formation. Similar to *Schistocerca* neuroblasts were divided into early, middle and late forming neuroblasts. Therefore the formation of neuroblasts has been divided into an early (NS4), middle (NS6 and NS8) and late forming stage (NS11). Comparing the assigned stages of *Tribolium* to the segregation waves in *Drosophila* demonstrates that *Tribolium* NS6 resembles a stage between S2 and S3 in *Drosophila*, NS8 resembles S4 and NS11 the last stage S5 in *Drosophila* (see figure 4-1.1).

The pattern of neuroblast formation in *Tribolium* is more reminiscent of the early, middle and late forming neuroblasts in *Schistocerca* and *Ctenolepisma* (Doe and Goodman 1985, Truman and Ball 1998) than the segregation in waves observed in *Drosophila* (Hartenstein and Campos-Ortega 1984). It is therefore possible that the continuous delamination of neuroblasts observed in *Tribolium*, *Schistocerca* and *Ctenolepisma* is a characteristic of their common insect ancestor, and that the delamination in waves observed in *Drosophila* is a more recently derived characteristic. However, it should be mentioned that although it is common practice to refer to five segregation waves in *Drosophila* it is questionable in how far this is an accurate description. Already Doe (1992)

observed intermediate patterns between segregation waves especially between the last three waves (S3 to S5) thus questioning the validity of distinct waves of segregation. Furthermore, Bossing *et al.* (1996b) noticed that individual neuroblasts vary in their time of delamination. For example NB 3-2 delaminates during S1 in nine, between S1 and S2 in six and during S2 in two out of 19 cases (Bossing *et al.* 1996). These observations suggest that the mode of neuroblast delamination in *Drosophila* may be more similar to the continuous delamination in *Schistocerca*, *Ctenolepisma* and *Tribolium* than previously assumed. It is therefore possible that at least some of the difference in the neuroblast delamination pattern observed between different insect species is due to methodological artefacts.

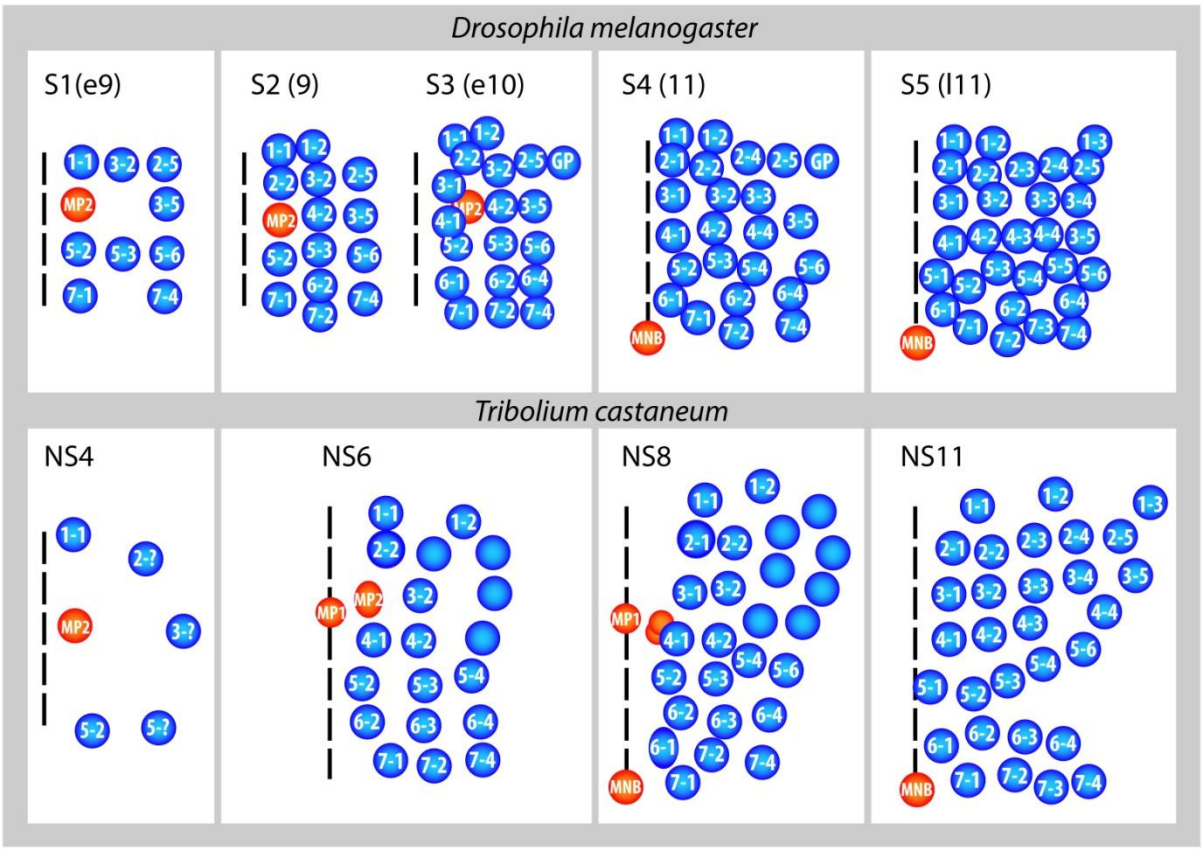


Figure 4-1: Neuroblast formation in *Drosophila* and *Tribolium*

The illustration shows the five segregation waves (S1 to S5) of *Drosophila* and the four established stages of neuroblast delamination in *Tribolium*. (*Drosophila*) Note that the first wave (S1) in *Drosophila* is further divided into early S1 and S1. As there is only one neuroblast added during S1 (NB 3-2) only S1 is shown. In brackets are the embryonic stages early (e); late (l). (*Tribolium*) Blank neuroblasts could only be designated in the final neuroblast arrangement. The two small orange circles depict the two neurons formed by MP2. The midline is indicated by the vertical dashed line.

Neuroblasts in *Tribolium* were designated according to their final position in the neuroblast pattern as adopted previously for other arthropods in which a neural precursor map has been established (Fig. 4-1.1 NS11). However, it is not possible to assign all neuroblast identities throughout neuroblast formation relying only on morphological cues (Fig. 4-1.1 NS4, NS6, NS8). Already in *Drosophila* it was demonstrated that neuroblasts are not necessarily formed in the same row and column, which they are eventually allocated to. For example the S1 NBs 1-1, 3-2 and 2-5 are all formed in the same row but with an increase in neuroblast numbers they are eventually positioned in three different rows (Fig. 4-1.1; Doe 1992, Broadus *et al.* 1995). Therefore, the first three stages established for *Tribolium* are first estimates based on similarities to *Schistocerca*, *Ctenolepisma* and *Drosophila* and have to be validated by molecular markers. Nevertheless, considering the similarities between all insects analysed so far the first delaminating neuroblasts in *Tribolium* most likely correspond to NBs 1-1, 2-5, 3-5, 5-2 and 7-4.

At stage NS6 the neuroblast array consists of three columns with six to seven neuroblasts each in *Tribolium* (Fig. 4-1.1). With the knowledge of the final neuroblast array and using the midline precursors MP1 and MP2 as landmarks (due to their characteristic position in a neuromere, see Results 3-1.5) the neuroblasts of the first column and most of the second column can be named according to their position. The next two stages (NS 8, NS11) are characterised by neuroblasts delaminating mostly in the anterior part of the hemineuromere. Additionally, neuroblast 6-1 and 5-1 can be unmistakably identified by their position as they both form closely along the midline at stage NS8 and NS11, respectively. Their time of formation is similar to the positionally equivalent *Drosophila* neuroblasts, where first the S3 NB 6-1 is formed and later the S5 NB 5-1 (Doe 1992). Furthermore, the median neuroblast (MNB) is formed at S4 in *Drosophila* and at NS8 in *Tribolium*.

After NS11 neuroblast numbers appear to decrease in *Tribolium*. In *Drosophila* neuroblasts become smaller after each division and eventually are not longer identifiable as neuroblasts (Hartenstein and Campos-Ortega 1984, Hartenstein 1987). Although not analysed in detail it appears that neuroblasts in *Tribolium* also loose volume over time. In contrast, in *Schistocerca* and *Ctenolepisma* neuroblasts degenerate during late stages of

embryogenesis as the formation of the adult nervous system in hemimetabolous insects is mostly completed in the embryo (Bate 1976, Doe and Goodman 1985, Shepherd and Bate 1990). In holometabolous insects in contrast there is a postembryonic phase of neurogenesis allowing the reorganisation of the larval nervous system to the adult nervous system (Prokop and Technau 1991, Booker and Truman 1987). Therefore it can be assumed that the loss of neuroblasts in *Tribolium* is the result of neuroblasts decreasing in volume and not the result of degeneration.

The allocation of specific identities to neuroblasts becomes problematic in row two and three in the anterior lateral part of the hemineuromere (Fig. 4-1, NS6 and NS8). The conserved pattern of *Drosophila*, *Schistocerca* and *Ctenolepisma* suggests that the most lateral neuroblasts in *Tribolium* (NBs 2-5, 3-5, 5-6, 7-4) form at stage NS4 and NS6 and all additional neuroblasts delaminate ventrally to them (Fig. 4-1.1 NS4 and NS6). This would suggest that the neuroblasts ventrally to them delaminate during NS6 and NS8 and would be NBs 2-3, 2-4, 3-3 and 3-4.

Further ambiguity exists regarding NBs 4-3, 4-4, 5-4, 5-5, 5-6 and 6-3. In *Drosophila* NB 4-4 is formed during S4 before NB 4-3 delaminates medially to it at S5. It was not possible to solve the sequence of NBs 4-3 and 4-4 formation.

Furthermore, in contrast to *Schistocerca*, *Ctenolepisma* and *Drosophila* which all exhibit six row five neuroblasts only five row five neuroblasts were observed in *Tribolium* (Fig. 4-2). The last neuroblast to be formed in *Ctenolepisma* and *Schistocerca* is neuroblast 5-5. Furthermore, also in *Drosophila* NB 5-5 is one of the last neuroblasts to delaminate during S5. In *Tribolium* four row five neuroblasts are formed by stage NS8 (see Fig. 4-1). The formation of NB 5-1 in the medial column next to the midline at stage NS11 appears to be conserved with *Drosophila* where it forms during the last segregation wave (S5). No further row five neuroblast appears to delaminate in *Tribolium*. Considering the temporal conservation of NB 5-5 formation between *Drosophila*, *Schistocerca* and *Ctenolepisma* it can be assumed that NB 5-5 is missing in *Tribolium*. Interestingly in *Schistocerca* and *Ctenolepisma* NB 5-5 is only formed in the thoracic segments but not in abdominal segments (Doe and Goodman 1985, Truman and Ball 1998, Shepherd and Bate 1990). The data for *Drosophila*, *Schistocerca* and *Ctenolepisma* suggest that NB 5-5 was part of

the neuroblast pattern in the common ancestor of insects and may have been lost in the coleopteran lineage.

In *Drosophila* NBs 5-2, 5-3 and 5-6 are all formed during the first segregation wave. At S4 NB 5-4 is formed and at S5 NBs 5-1 and 5-5 delaminate. In *Tribolium* neuroblast 5-2 is already identifiable in the first two stages. Assuming the existence of a generally conserved sequence in which neuroblasts are formed in insects, the neuroblast formed lateral to NB 5-2 and NB 5-3 at stage NS6 should be the equivalent of the *Drosophila* NB 5-6. However, analysis of *Tc-msh* and *Tc-wg* expression in *Tribolium*, as presented in Chapter 3.1 and 3.3, indicates that this may not be the case. At stage NS6 the most lateral neuroblast of row five expresses *Tc-msh* and *Tc-wg*. It appears that this neuroblast maintains the expression of both genes over neurogenesis and that a further row five neuroblast delaminates laterally to it which is neither *Tc-wg* nor *Tc-msh* positive (Fig. 4-3 and 4-4). This suggests that in contrast to *Drosophila* NB 5-6 is formed at a later stage (NS8) after NB 5-4 has already delaminated.

In *Tribolium* four row six neuroblasts are formed, whilst in *Drosophila* only three, with NB 6-3 missing. In contrast, C*tenolepisma* and *Schistocerca* both form NB 6-3 (Fig. 4-2). Neuroblast 6-3 generates a noticeable larger lineage in *Ctenolepisma* than in *Schistocerca* (Truman and Ball 1998). Truman and Ball (1998) speculate that NB 6-3 might generate interneurons connected to the cercal sensory system that is “highly developed in silverfish, reduced in grasshopper and absent in flies” (Edwards 1997). *Tribolium* has cerci at the last abdominal segment (Hayashi 1966) which would support the suggestion by Truman and Ball (1998) that NB 6-3 generates interneurons connected to the cercal system. Further analysis is required in order to make firm conclusions regarding the lineage size of NB 6-3 in *Tribolium* and the fate of NB 6-3 progeny.

In addition to neuroblasts the glial precursor cell (GP) is formed in *Drosophila* during S3 as the most lateral cell in the neuroblast array (Fig. 4-1.1; Doe 1992). It divides symmetrically before S5 neuroblasts delaminate and can be identified by the expression of the two marker genes *ftz* and *mirr* (Doe 1988a, Broadus *et al.* 1995). In the present work neither the expression of these genes nor the division pattern of neuroblasts was analysed. Furthermore, lateral neuroblasts of row two and three could not be distinguished by position alone. Therefore it was not possible to characterise the GP. Yet,

as *Schistocerca* also possesses a GP (Doe and Goodman 1985) its presence in *Tribolium* is considered likely.

In summary, the findings of the current study support the idea that the arrangement of neuroblasts along the AP and DV axis and the relative time of formation of positionally equivalent neuroblasts are largely conserved between insects. Nevertheless, some subtle changes regarding neuroblast numbers and relative time of formation were observed. In case of row six neuroblasts, *Tribolium* may represent the ancestral state forming four row six neuroblasts as *Schistocerca* and *Ctenolepisma*. In contrast the loss of NB 6-3 in *Drosophila* may represent a derived stage. However, in row five *Drosophila*, *Schistocerca* and *Ctenolepisma* all possess six neuroblasts, suggesting that the reduced number of five row five neuroblasts in *Tribolium* is a derived characteristic (Fig. 4-2).

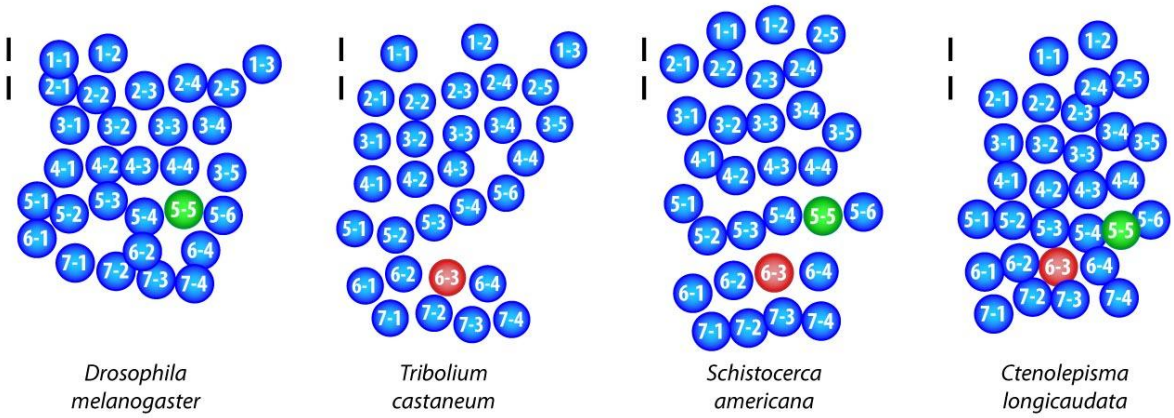


Figure 4-2: Neuroblast maps in different insects

Final arrangement of neuroblasts in *Drosophila*, *Tribolium*, *Schistocerca* and *Ctenolepisma* in a single hemineuromere. All maps based on schematic drawings from the following publications (Doe and Goodman 1985, Doe *et al.* 1992, Truman and Ball 1998) except for *Tribolium* which is based on the present work. In all insects there are seven rows with two to six columns of neuroblasts formed. *Drosophila*, *Schistocerca* and *Ctenolepisma* all possess NB 5-5 (green) in contrast to *Tribolium*. NB 6-3 is missing in *Drosophila* but present in *Tribolium*, *Schistocerca* and *Ctenolepisma* (red). The midline is indicated by the black dashed line.

Expression pattern of segmentation genes differs substantially to *Drosophila*

The present work demonstrates that changes in the expression of genes involved in conferring unique identity to neuroblasts in *Tribolium* and *Drosophila* are one mechanism by which evolutionary changes to the nervous system may occur.

Several of the genes conferring neuroblast identity in *Drosophila* have an earlier function in segmentation and are known as segmentation genes (reviewed in Peel *et al.* 2005). Some of these genes have been analysed for their role during segmentation in *Tribolium* but nothing is known about their function during neurogenesis (Brown *et al.* 1994a, b and c, Nagy and Carroll 1994, Brown and Denell 1996, Brown *et al.* 1997, Choe and Brown 1998, Choe *et al.* 2006, Farzana and Brown 2008).

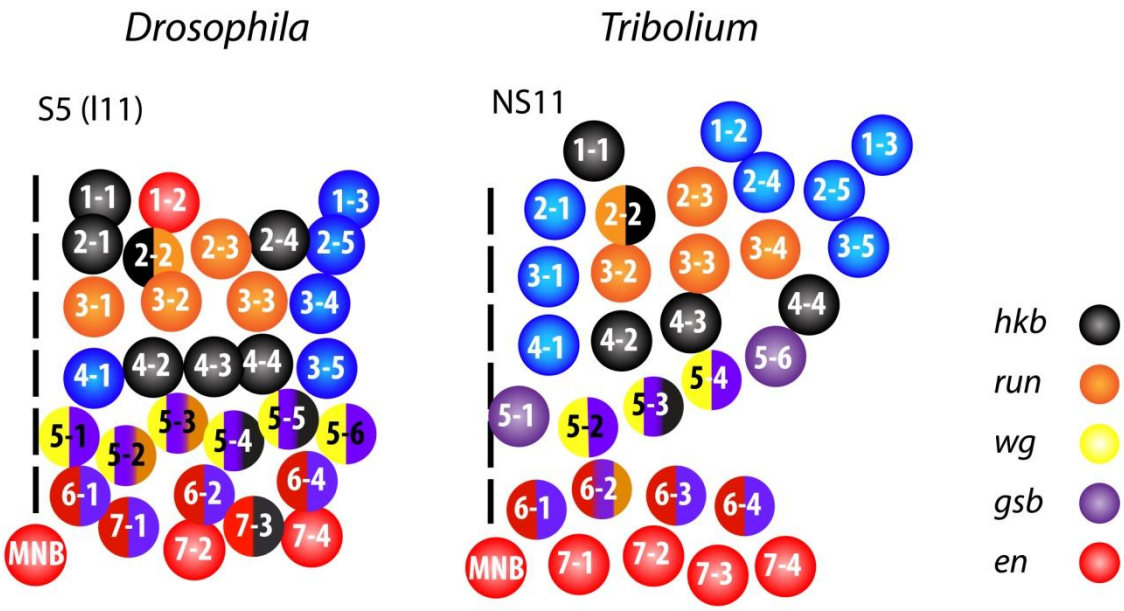


Figure 4-3: Comparison of neural identity gene expression in *Drosophila* and *Tribolium*

Illustrated is the final neuroblast map for *Drosophila* (S5) and *Tribolium* (NS11) depicting gene expression in neuroblasts. Note that the overall gene expression repertoire of individual neuroblasts is presented and not necessarily all genes are still expressed at this stage. Neuroblasts which do not express any of the given genes are coloured in blue. The midline is indicated by the black dashed line. Late embryonic stage 11 (l11).

The expression pattern of the segment polarity gene *engrailed* in the nervous system has been intensively studied in species of all arthropod lineages, and exhibits a high degree of conservation in its expression pattern (Duman-Scheel and Patel 1999, Patel 1989, 1994, Stollewerk and Chipman 2006, Fabritius-Vilpoux *et al.* 2008). Its expression in the neuroectoderm, neuroblasts and progenitor cells of *Tribolium* has been described previously but has not been related to specific neuroblasts (Brown *et al.* 1994). Using the map established in the current work now permits the assignment of *Tc-en* expression to individual neuroblasts (Fig. 4-3). As anticipated, *Tc-en* is expressed in all row six and seven neuroblasts. According to the map established here *Tc-en* is not expressed in NB 1-2. This finding is somewhat unexpected, as in both *Schistocerca* and *Drosophila* NB 1-2 expresses *engrailed* (Doe 1992, Broadus and Doe 1995). Furthermore, in crustaceans, chelicerates and myriapods it is expressed in row one, six and seven neuroblasts/NPGs (Duman-Scheel and Patel 1999, Patel 1989, 1994, Fabritius-Vilpoux *et al.* 2008). In chelicerates and myriapods *en* expression in row one even expands to several neural precursor groups (Stollewerk and Chipman 2006). However, it is possible that *Tc-en* is expressed transiently in NB 1-2 whilst still in the neuroectoderm as it is appears possible that neuroectodermal cells out of which NB 1-2 arises express *Tc-en*. Therefore, a scenario where NB 1-2 expresses *Tc-en* only transiently at the very beginning is possible.

A marker gene for row five and six neuroblasts in *Drosophila* is *gooseberry distal* (*gsb-d*) (Fig. 4-3; Doe 1992). *gsb-d* is expressed in the neuroectoderm in the area out of which row five and six neuroblasts arise and in all row five and six neuroblasts. Additionally NB 7-1 expresses *gsb-d* (Gutjahr *et al.* 1993). The expression pattern in the neuroectoderm and neuroblasts is conserved between *Tribolium* and *Drosophila*. However, in addition to *Tc-gsb* expression in the neuroectoderm and neuroblasts expression was detected in neurons and axons. The expression of *gsb-d* in axons has not been described in *Drosophila* and may represent a divergent character in beetles.

In *Drosophila* the signalling molecule *wingless* is expressed in neuroectodermal cells of row five and all row five neuroblasts from the time of their formation until the end of neurogenesis (Fig. 4-3; Doe 1992; Chu-LaGraff and Doe 1995). Previous research on the expression of *Tc-wg* during segmentation demonstrated that the expression in stripes in the ectoderm is conserved between *Drosophila* and *Tribolium* (Nagy and Carroll 1994).

The finding that in *Tribolium wg* expression is confined to three of the five row five neuroblasts was therefore unexpected. Furthermore, in contrast to *Drosophila Tc-wg* expression in *Tribolium* is transient, with NB 5-2 ceasing expression shortly after its formation, followed by NB 5-3 and eventually NB 5-4. In *Drosophila* the protein product of *wingless* segregates into neuroectodermal cells out of which row four and six neuroblasts arise. Loss of *wg* results in less neuroblasts being formed or changes in their identity (Chu-LaGraff and Doe 1993). Given that *Tc-wg* is initially expressed in neuroectodermal cells along the whole of row five could mean that the mechanism of conferring row four and six neuroblast identity is conserved between *Tribolium* and *Drosophila*. The lack of *Tc-wg* expression later in row five neuroblasts may result in the generation of different lineages in these row five positionally homologous neuroblasts in *Drosophila* and *Tribolium*.

In addition to the segment polarity genes, the expression patterns of two segmentation genes, the pair-rule gene *runt* and the gap gene *huckebein*, were analysed which are involved in conferring neural identity to neuroblasts in *Drosophila*.

In *Drosophila runt* is partially expressed in neuroblasts of row two and three and therefore an appropriate marker gene for some row two and three neuroblasts (Fig. 4-3). Furthermore, *runt* is expressed in row five neuroblasts (Dormand and Brand 1998). The expression of *Tc-run* in *Tribolium* was analysed in NS6 and NS8 embryos and exhibited similarities of expression in row two and three neuroblasts when compared to *Drosophila*. It appears that it is expressed in NBs 2-2, 2-3, 3-2, 3-3 and 3-4. Additionally the midline precursor cell MP1 expresses *Tc-run* as in *Drosophila*. In *Drosophila run* is known as an activator of *even-skipped* expression in progeny of NB 3-3 and is necessary for *eve* expression in the EL neurons. (Duffy *et al.* 1991, Dormand and Brand 1998). In *Tribolium Tc-run* is expressed in many neurons, some of which are positioned in the anterior lateral domain of the hemineuromere where the neurons of the EL cluster are formed. Assuming that *Tc-run* regulates Eve expression in the same way as in *Drosophila*, the presence of the EL cluster in *Tribolium* (see results chapter 3-3) would support a homology of NB 3-3 in *Tribolium* and *Drosophila*. However, it is also possible that in *Tribolium* a different neuroblast generates the neurons of the EL cluster. It will therefore be very interesting to perform RNAi silencing studies of *Tc-run* and analyse the effect on Eve expression.

A major difference between *run* expression in *Drosophila* and *Tribolium* occurs in posterior rows. In *Drosophila run* is expressed in two row five neuroblasts, whilst in *Tribolium* no expression in row five neuroblasts was detected. Instead neuroblast 6-2 expresses *Tc-run*.

Expression of the gap gene *hkb* appears to be partially conserved between *Tribolium* and *Drosophila*. Comparing the results obtained for *Tribolium* with *Drosophila* demonstrates that the early expression in neuroblasts of row four is similar (Fig. 4-3). In row five both in *Tribolium* and *Drosophila* NB 5-4 expresses *hkb*. Yet, NB 5-3 positioned medially to NB5-4 expresses *Tc-hkb* in *Tribolium*, whereas in *Drosophila* NB 5-5 expresses *hkb*. A further noticeable difference occurs in row two neuroblasts. In *Tribolium* only one row two neuroblast expresses *hkb*, whilst in *Drosophila* there are three hkb^{+} neuroblasts in row two. Additionally, NB 7-3 does not express *hkb* in *Tribolium* but in *Drosophila*. This is interesting as Lundell *et al.* (1996) demonstrated that *hkb* and *en* are uniquely co-expressed in serotonin neurons, which are generated by NB 7-3 (Schmid *et al.* 1999). Loss of either results in loss of serotonin expression and pathfinding defects. Furthermore, NB 7-3 in *Schistocerca* and *Drosophila* appears to be a genuine homologue as it not only shares the relative same position but also generates the only two serotonin neurons in both species (Taghert *et al.* 1984, Lundell *et al.* 1996). However, nothing is known about *hkb* expression in *Schistocerca*. Assuming that also in *Tribolium* NB 7-3 generates serotonin neurons the lack of *hkb* suggests that *hkb* is not required for the correct specification of serotonin neurons in *Tribolium*. However, expression was detected in neuroectodermal cells of row seven. Therefore it is possible that the late forming NB 7-3 may express *hkb* whilst still in the neuroectoderm. Analysing the lineage of NB 7-3 will reveal in how far the lineage is conserved between NB7-3 in *Drosophila* and *Tribolium*. Neuroblast 7-3 may be an example of a homologous neuroblast in *Tribolium* and *Drosophila* that maintains a part of its neural lineage, although the gene expression profile has diverged. Alternatively serotonin neurons may be generated by a different neuroblast. However, as there was no neuroblast found co-expressing *Tc-en* and *Tc-hkb* in *Tribolium* this would again imply that *Tc-hkb* is not involved in specifying serotonin neurons in *Tribolium*.

In contrast expression in NB 1-1 appears to be conserved. In *Tribolium hkb* expression in NB 1-1 only commences at stage NS8, probably long after it has delaminated. This is similar to *Drosophila* were *hkb* expression in NB 1-1 does not commence simultaneously with its delamination at S1 but only after all S3 neuroblasts have delaminated (Chu-LaGraff *et al.* 1995). Furthermore, *hkb* expression in NBs 2-2 and 4-2 appears to be conserved. Detailed functional analyses for NBs 1-1, 2-2 and 4-2 in *Drosophila* demonstrated that loss of *hkb* does not alter neuroblast identity, as the neuroblasts maintain the expression of characteristic marker genes (Chu-LaGraff *et al.* 1995, Bossing *et al.* 1996a). Nevertheless, changes and disruptions in axon pathfinding and formation of glial cells were observed. For example, neuroblast 4-2 in *Drosophila* generates the Eve^+ GMC (4-2a), which in turn generates the Eve^+ RP2 and RP2 sibling neurons. In *hkb* mutants these cells are formed but do not express Eve. This lack of Eve expression eventually results in axon pathfinding defects (Chu-LaGraff *et al.* 1995). The expression of *even-skipped* in the RP2 neurons appears to be conserved between insects and between crustaceans (Duman-Scheel and Patel 1999). Therefore it may be concluded, that *hkb* expression in NB 4-2 may be part of the molecular ground pattern in the last common ancestor of insects and possibly even crustaceans.

The comparison of gene expression patterns of individual neuroblasts in the present work demonstrates that there are differences regarding the expression of neural identity genes, which confer spatial identity along the AP axis. For example, the segment polarity gene *wingless*, the pair-rule gene *runt* and the gap gene *hkb* exhibit a partially conserved expression pattern but also show notable differences. The question remaining is whether and how these changes influence neuroblast identity in *Tribolium*. Whilst differences in gene expression of neuroblasts may not necessarily result in changes in the molecular profile of their progeny they may, however, lead to alterations of the axonal projections which in turn may cause changes to the neuronal network observed between different insect species. Alternatively, functional studies may reveal that, although the expression pattern varies, similar lineages are formed, due to changes in the gene regulatory network.

The process of identifying homologous individual neuroblasts in different insects is not trivial. Position alone is not sufficient to identify homologous neuroblasts with the degree

of confidence that lineage analyses in *Schistocerca* and *Drosophila* has provided (Broadus *et al.* 1995). For example NB 1-1 in *Drosophila* was initially named NB 2-2 according to its previously identified positionally equivalent neuroblast in *Schistocerca*. Lineage analysis revealed that NB 2-2 generates the aCC/pCC neurons in *Drosophila* whereas in *Schistocerca* these neurons are formed by NB 1-1. To reflect the similarities in lineage formation NB 2-2 was renamed NB 1-1 in *Drosophila*. Furthermore, the cell initially named NB 1-1 was renamed NB 1-2, as it is the only *en* expressing neuroblast in the anterior row both in *Drosophila* and *Schistocerca*.

Although data on neuroblast lineages was not obtained in the present work, the combination of time of formation, position and gene expression studies permits speculation regarding which positionally equivalent neuroblasts in *Tribolium* may be homologous neuroblasts of those in *Drosophila*. The data collected in the present work suggest that NBs 2-2, 3-2, 4-2, 5-1, 5-2, 6-1, 6-4 and 7-1 may be homologues (see table 4-1) with NB 5-2 exhibiting the greatest similarities with its positionally equivalent neuroblast in *Drosophila*. In both insects, it is one of the first neuroblasts to delaminate, to express *svp* and furthermore, expresses *gsb, wg* and *vnd*. Neuroblast 2-2 is formed after the early forming neuroblasts and expresses *hkb* from its formation onwards and furthermore, *runt* in both *Tribolium* and *Drosophila*. Neuroblast 5-1, however, illustrates the limitations of identifying homologous neuroblasts on the level of position, time of formation and gene expression only. In both insects it is formed late, at the same position and expresses *gsb* and *vnd*. Yet, neuroblast 5-1 does not express *wg* in *Tribolium* but does so in *Drosophila*. This, however, does not necessarily suggest that the two neuroblasts are not genuine homologues. It may be the case, as discussed above, that this neuroblast has slightly altered its gene expression profile over evolution, thus generating changes in the species specific neuronal network.

Table 4-1: Homologous neuroblasts in *Tribolium* and *Drosophila*

Putative homologous neuroblasts in *Tribolium* and *Drosophila*, based on their position, time of formation and expression of neuroblast identity genes. In the first column the neuroblast identity is provided. The second column shows, firstly the time of formation and secondly summarises the expression of the neuroblast identity genes investigated.

Neuroblast	*Tribolium*	*Drosophila*
5-2	NS4 (early) *gsb, wg, vnd* first NB to express *svp*	S1 (early) *gsb, wg, vnd* first NB to express svp
2-2	S2 (middle) *hkb, run*	NS6 (middle) *hkb, run*
3-2	S2 (middle) *run*, *ind*	NS6 (middle) *run, ind*
4-2	S2 (middle) *hkb*, *ind*	NS6 (middle) *hkb*, *ind*
5-1	S5 (late) *gsb, vnd, wg*	NS11 (late) *gsb, vnd*
6-1	S3 (middle) *gsb, en*, *vnd*	NS8 (middle) *gsb, en*, *vnd*
6-4	S3 *gsb, en, msh*	NS6 *gsb*, *en*, *msh*
7-1	S1 (early) *gsb, en, vnd*	NS6 (middle) *gsb, en, vnd*

In *Schistocerca* it has previously been demonstrated that the expression pattern in early neuroblasts is highly conserved in comparison to *Drosophila*. Evolutionary changes in gene expression do occur in later forming neuroblasts (Broadus and Doe 1995). The present work demonstrates that this is also partially the case in *Tribolium*. *Tc-hkb* expression in the early formed NBs 1-1 and 4-2 for example is conserved, whilst differences are observed in the late forming neuroblasts 2-1 and 7-3. It is conceivable that early neuroblast identities are somewhat constrained because some of these neuroblasts generate the pioneer neurons which set up the highly conserved axonal scaffold (Thomas *et al.* 1984).

Temporal gene expression is conserved between *Tribolium* and *Drosophila*

The temporal genes confer temporal identity to neuroblasts, after they have delaminated. Several genes are expressed sequentially in every neuroblast over its lifespan, allowing the formation of neurons and glial cells with differing fates (see review Pearson and Doe 2004, Brody and Odenwald 2005). Regulatory interactions between the temporal genes facilitate the switch from one gene to another. The expression of a given temporal gene is maintained in the respective progeny formed by a neuroblast at the time of its expression. In *Drosophila* five genes form the major gene cascade, beginning with *hb* expression in newly formed neuroblasts and proceeding with *Kr*, *nub*, *cas*, *grh* expression (Brody and Odenwald 2005). The change of expression from one temporal gene to the next happens after one or two neuroblast divisions, resulting in neuronal layers with distinct gene expression (Isshiki *et al.* 2001). Basally positioned, early born, neurons express *hb* and *Kr*, middle layer neurons express *nub* and late apical neurons express *cas* and/or *grh*. Only a few neuroblasts in *Drosophila* have been analysed in detail, which has nonetheless demonstrated that not every neuroblast necessarily expresses all genes (Isshiki *et al.* 2001, Novotny *et al.* 2002). In *Tribolium* nothing was known about the expression of temporal genes in the nervous system prior to the current work. This work demonstrates that as in *Drosophila* the first gene expressed in most neuroblasts is *Tc-hb*, followed or accompanied by *Tc-Kr*. Subsequently *Tc-nub* expression is detected, closely followed by *Tc-cas* expression. The expression of *grainy head* was not analysed. Temporal genes in *Drosophila* partly regulate their expression by negative and positive feedback, although it appears that more genes may be involved in the process. For example, *svp* was found to provide the switch from *hb* expression to *Kr* in *Drosophila* (Kanai *et al.* 2005). Loss of *svp* results in a loss of late lineage GMCs, which are specified by *nub*. In wild type *Drosophila hb* expression is switched off by *svp*. Without *svp* expression *hb* expression persists and represses *nub* expression (Kanai *et al.* 2005). In *Tribolium* the expression of *Tc-svp* could not be analysed in detail due to unsatisfying *in situ* hybridisation results. It was, however, possible to demonstrate that the first neuroblast expressing *Tc-svp* is NB 5-2 as in *Drosophila* (Doe 1992). Furthermore, in *Schistocerca* NB 5-2 is also the first neuroblast to express *svp* and a conserved *svp* expression in several neuroblasts between *Drosophila* and *Schistocerca* has been previously observed

(Broadus and Doe 1995). In *Tribolium* increasing numbers of neuroblasts commence *Tc-svp* expression during development. Detailed analysis of the expression pattern, on the basis of single neuroblasts, was unfortunately not possible. On the basis of the results presented here, and the expression pattern in *Drosophila* and *Schistocerca*, it is suggested that *Tc-svp* expression is mostly conserved and the function of the nuclear orphan receptor *svp* might be similar in all three insects.

Further research should be performed regarding the exact temporal gene expression pattern of individual neuroblasts, specifically NBs 7-1, 7-3, 7-4, 6-4 or 2-4, which have been analysed in detail in *Drosophila* (Kambadur *et al.* 1998, Isshiki *et al.* 2001; Novotny *et al.* 2002), in order to draw further conclusions. Additionally loss of function studies should reveal if the temporal genes show similar interactions as those observed in *Drosophila*.

In arthropods other than *Drosophila* only very limited data on temporal gene expression and function is available. The only such study conducted to date analysed the expression of *hb* and *Kr* in the myriapod *Strigamia maritima* (Chipman and Stollewerk 2006). Although different types of neural precursor cells are present in the myriapods, a temporal sequence in the expression of *hb* and *Kr* was observed. This suggests that the mechanism through which neuroblasts confer different identities to their progeny may be conserved within arthropods.

Although the results presented in the current work are only a first step in analysing temporal gene expression, they are compatible with the idea that the overall expression of temporal genes is conserved in insects. Therefore, the mechanisms which confer temporal identity to neuroblasts permitting the formation of neurons and glial cells with unique fates appear to be conserved between insects and may even be conserved within arthropods.

Columnar gene expression experienced significant changes over time

The data currently available on columnar gene expression and function in different groups of arthropods suggest that the division of the neuroectoderm into three columns along the DV axis is conserved amongst arthropods (Skeath 1999: *Drosophila,* Wheeler *et al.* 2005: *Tribolium*, Doeffinger and Stollewerk 2010: chelicerates, myriapods). However, Doeffinger and Stollewerk (2010) demonstrated that subtle differences in the expression pattern of columnar genes exist between chelicerates and myriapods, and that these differences are even greater in comparison to insects. They suggested that changes in the expression pattern of columnar genes in individual NBs/NPGs may be one mechanism producing variation in nervous systems.

Furthermore, it appears that in insects columnar gene expression also varies to some degree. Wheeler *et al.* (2005) describe subtle changes in the expression pattern of columnar genes between the two insects *Drosophila* and *Tribolium*. These authors were, however, lacking a neuroblast map for *Tribolium* and therefore could not compare columnar gene expression in single neuroblasts. In the present work further differences on the level of individual neuroblasts were revealed, thereby demonstrating that changes of spatial gene expression along the DV ventral axis may be an additional mechanism for generating diversity within insect nervous systems.

vnd expression is partially conserved between insects

Wheeler *et al.* (2005) concluded that the expression and function of *vnd* in *Tribolium* and *Drosophila* are very similar. Further analysis, however, demonstrates that the expression pattern is not as conserved as previously assumed. The early expression in two continuous columns before gastrulation is similar between the two species (Jimenez *et al.* 1995, Mellerick and Nirenberg 1995, Wheeler *et al.* 2005). After gastrulation, however, significant differences are noticeable. In *Drosophila vnd* is expressed continuously in the medial neuroectoderm before the first neuroblasts delaminate and only ceases after S2 neuroblast delamination (Chu *et al.* 1998). This is in contrast to *Tribolium*, where expression in the neuroectoderm ceases in the anterior and posterior part of a hemineuromere prior to the first neuroblasts delaminating. Eventually the expression extends again along the AP axis so that all ventral neuroblasts expressing *Tc-vnd* arise

from *Tc-vnd* neuroectodermal cells. In both insects neuroblasts 3-1, 4-1, 5-1, 5-2, 6-1, 6-2, 7-1 and the midline precursor MP2 express *vnd*. Differences occur in the case of neuroblasts 1-1, 1-2, 2-1, 2-2 and 7-2. Although neuroblast 1-1 arises from a cluster of *Tc-vnd* expressing neuroectodermal cells no expression in the neuroblast was detected in *Tribolium* after delamination. Therefore, it seems that NB 1-1 expresses *Tc-vnd* during delamination and then immediately ceases expression. This might be sufficient for obtaining the specific identity cues required to establish its unique fate. Furthermore, whilst *Tc-vnd* transcript might no longer be detectable at this point, the protein may still be present in NB 1-1. In *Drosophila*, for example, Vnd protein is still detectable after *vnd* transcript has already ceased to be expressed (Chu *et al.* 1998, McDonald *et al.* 1998).

A noticeable difference in *vnd* expression between the two insect species is observed in the case of NBs 1-2 and 2-2, neither of which expresses *Tc-vnd* in *Tribolium* but which both do so in *Drosophila*. Some ambiguity exists in the interpretation of data regarding NBs 7-2 and 2-1 in the present work. In *Drosophila* NB 7-2 does not express *vnd* initially but only commences its expression after all neuroblasts have been formed (Chu *et al.* 1998). In *Tribolium* in stages older than NS11 there are two neuroblasts in row seven, adjacent to each other, both of which express *Tc-vnd*. Assuming homology with *Drosophila* in neuroblast position the lateral one may be NB 7-2. Furthermore, it may be that at the same stage the most anterior ventral neuroblast expressing *Tc-vnd* is NB 2-1. However, due to decreasing numbers of recognizable neuroblasts after NS11, subsequent movement of neuroblasts and a lack of marker genes it is not possible to unequivocally assign these neuroblasts a specific identity.

Tc-ind expression exhibits greater similarities with myriapods than with *Drosophila*

ind is expressed in the intermediate column neuroectoderm and neuroblasts in *Drosophila* (Weiss *et al.* 1998). The present work demonstrates evolutionary modifications in the expression of *Tc-ind* in the neuroectoderm and late expression in neuroblasts of *Tribolium* compared to *Drosophila*. The earliest difference visible between the two species is the continuous expression of *ind* lateral to *vnd* in a column, commencing already in the blastoderm, in *Drosophila*. In *Tribolium Tc-ind* is not expressed prior to gastrulation (Wheeler *et al.* 2005). With the beginning of neuroblast formation in *Drosophila ind* expression is detected in the entire intermediate column neuroectoderm and the two

intermediate S1 neuroblasts (NBs 3-2 and 5-3). *ind* expression becomes restricted to intermediate column neuroblasts (NBs 3-2, 4-2, 5-3, 6-2 and 7-2) and ceases in the neuroectoderm. In *Tribolium*, in contrast, *Tc-ind* expression in the intermediate neuroectoderm only commences shortly before the first neuroblasts delaminate and is not expressed along the entire intermediate column neuroectoderm. This expression results in a gap of non-expressing neuroectodermal cells in the anterior part of each segment. Interestingly, although initially in *Drosophila* all neuroectodermal cells along the intermediate column express *ind*, neuroblasts of row one and two also do not express *ind* at any time (Weiss *et al.* 1998). Furthermore, the intermediate NBs 3-2, 4-2 and 5-3 express *ind*, both in *Drosophila* and *Tribolium*.

In *Drosophila ind* expression becomes restricted to NBs 6-2 and 7-2 at stage S3. By stage S4, only NB 6-2 continues to express *ind* (Weiss *et al.* 1998). Wheeler *et al.* (2005) observed a similar pattern in *Tribolium*, with *Tc-ind* expression becoming confined to two posterior neuroblasts. In the present work these two neuroblasts were identified as NBs 5-3 and 6-3. Additionally it was shown in the present work, that the expression of *Tc-ind* becomes very weak in NB 5-3 around stage NS8. Yet, at stage NS 11, expression in NB 5-3 is increasing again and *Tc-ind* is detected in the late forming NB 7-3 and in a single row three neuroblast. This late expression has not been described in *Drosophila*. Furthermore, in *Drosophila* the two most posterior *ind*$^{+}$ neuroblasts are NBs 6-2 and 7-2, whereas in *Tribolium* they are NBs 6-3 and 7-3. As mentioned above, NB 6-3 is missing in *Drosophila*, whilst it is present in *Tribolium*, *Schistocerca* and *Ctenolepisma*, however, *ind* expression has not been analysed in the latter insect species. In addition, there is a difference in the expression of the columnar genes in the two posterior *ind*+ neuroblasts in *Tribolium* and *Drosophila*. In *Drosophila* NBs 6-2 and 7-2 first express *ind* and then switch to *vnd* expression (Weiss *et al.* 1998, Chu *et al.* 1998) whilst this is not the case in *Tribolium*. The way in which these changes influence neuroblast identity of positionally equivalent neuroblasts can only be addressed by lineage analyses and the expression pattern of further marker genes.

Data on *ind* expression in arthropods other than insects is only available for the myriapod *Glomeris marginata* (Dove 2003, Doeffinger 2010). As in *Tribolium*, but in contrast to the initial expression in *Drosophila*, *ind* in *Glomeris* is not expressed continuously along the

neuroectoderm. Instead, expression is restricted to NPGs in the posterior part of a segment.

These data suggest that the continuous expression of *ind* in the intermediate column of the neuroectoderm in *Drosophila* is a derived character. Furthermore, in *Glomeris ind* is expressed in lateral neural precursor groups. In *Tribolium Tc-ind* is also expressed in the more lateral NBs 6-3 and 7-3 than in *Drosophila* (NBs 6-2 and 7-2), again suggesting that this may be a derived characteristic in *Drosophila*. In contrast the up-regulation of *ind* in later stages has only been reported in *Tribolium* and may thus be a characteristic specific to the *Tribolium* or coleopteran lineage.

Early forming neuroblasts express *Tc-msh*

In the present work differences in *msh* expression were observed at the level of neuroectoderm expression and neuroblasts between *Tribolium* and *Drosophila*. In *Drosophila msh* expression in the neuroectoderm is characterised by two phases of expression. Initially *msh* expression begins prior to neuroblast formation in clusters of ectodermal cells. The expression extends and eventually forms a continuous expression domain along the AP axis of the entire embryo, corresponding to the dorsal/lateral domain of the neuroectoderm (Lord *et al.* 1995, D'Alessio and Frasch 1996, Isshiki *et al.* 1997). The initial expression lasts until S1 neuroblasts have delaminated. Shortly before S3-S5 neuroblasts delaminate *msh* expression is re-initiated in many lateral proneural clusters out of which lateral neuroblasts arise (Isshiki *et al.* 1997).

Wheeler *et al.* (2005) claim that '*Tc-msh* shares the late but not early phase of *msh* expression pattern with *Drosophila'* and therefore suggest that early forming neuroblasts are independent of *Tc-msh* expression, whereas late-forming neuroblasts rely on *Tc-msh* to acquire lateral cell fate, as is the case in *Drosophila*. However, the data obtained in the present work stands in stark contrast to these findings. According to the present work *Tribolium Tc-msh* expression begins in ectodermal cells outside the neuroectoderm with the expression eventually extending into the lateral part of the neuroectoderm before the first neuroblasts delaminate. Nevertheless, early forming lateral neuroblasts already delaminate from *Tc-msh*+ neuroectodermal cells and express *Tc-msh* similar to *Drosophila*. These early *Tc-msh*+ neuroblasts most likely belong to row two and five.

Unlike in *Drosophila*, however, *Tc-msh* is never expressed in the entire lateral domain and is concentrated on neuroectodermal cells out of which *Tc-msh* expressing neuroblasts arise. Interestingly, only one of the early delaminating lateral neuroblasts (NB 7-4) in *Drosophila* strongly sustains *msh* expression, whilst the other three neuroblasts appear to switch off *msh* expression shortly after delamination, with one neuroblast (NB 2-5) reinitiating expression one stage later. In the present work it was not possible to unequivocally confirm whether the early *Tc-msh* expressing neuroblasts maintain *Tc-msh* expression and eventually become recognizable in the final neuroblast pattern as row two and five neuroblasts. Furthermore, *Tc-msh* expression appears to be continuous in *Tribolium* in contrast to the two phases observed in *Drosophila*.

Comparing *msh* expression in the final map reveals further differences and similarities between *Drosophila* and *Tribolium* (Fig. 4-3). For example the most lateral neuroblasts of row six and seven (NBs 6-4 and 7-4) express *msh* in both insects. However, both in row four and five, there are two neuroblasts expressing *Tc-msh* (NBs 4-2, 4-3, 5-3, 5-4) whilst in *Drosophila* only NBs 4-3 and 5-4 are *msh* positive. Furthermore, neuroblasts 4-2 and 5-3 co-express *Tc-msh* and *Tc-ind*. In *Drosophila* no co-expression of *ind* and *msh* has been observed.

Ambiguity exists regarding which specific neuroblasts in row two and three express *Tc-msh* during those stages prior to the delamination of all neuroblasts (NS6, NS8). It appears that NBs 3-2 and 2-2 express *Tc-msh* at stage NS8. At stage NS11, however, these cells are clearly negative for *msh* expression. The lack of neuroblast markers for row one, two and three does not permit the assignment of *Tc-msh* expression to specific neuroblasts. Further analysis concerning the exact formation of neuroblasts in these rows would be required before such assertions could be made. Nevertheless comparing *msh* expression in the final neuroblast arrangement demonstrates that in *Drosophila* the most lateral neuroblasts of row two and three express *msh* (NBs 2-4, 2-5, 3-4) whilst in *Tribolium* neuroblasts further ventrally (NBs 2-3, 3-3) express *Tc-msh* with two more neuroblasts positioned laterally to them (Fig. 4-3).

In chelicerates and myriapods *msh* is expressed in lateral neural precursor groups (NPGs) as in *Drosophila* (Doeffinger and Stollewerk 2010). However, in contrast to *Drosophila* all NPGs arising from the *msh* domain express *msh* in both chelicerates and

myriapods. Therefore, early expression along the entire lateral domain of the neuroectoderm in *Cupiennius* and *Glomeris* is more reminiscent of *Drosophila* than of *Tribolium*. In *Drosophila* expression along the entire lateral domain ceases after S1 neuroblasts have delaminated, whilst in *Cupiennius and Glomeris* this is not the case. Expression of *msh* along the entire lateral domain of the neuroectoderm may be the ancestral state in arthropods. In insects the role of *msh* appears to have diverged with not all lateral neuroblasts being under the influence of *msh*. *Tribolium* may have diverged even further from the ancestral state, in comparison to *Drosophila*, by diminishing expression in the neuroectoderm only to cell clusters of which *msh* expressing neuroblasts arise.

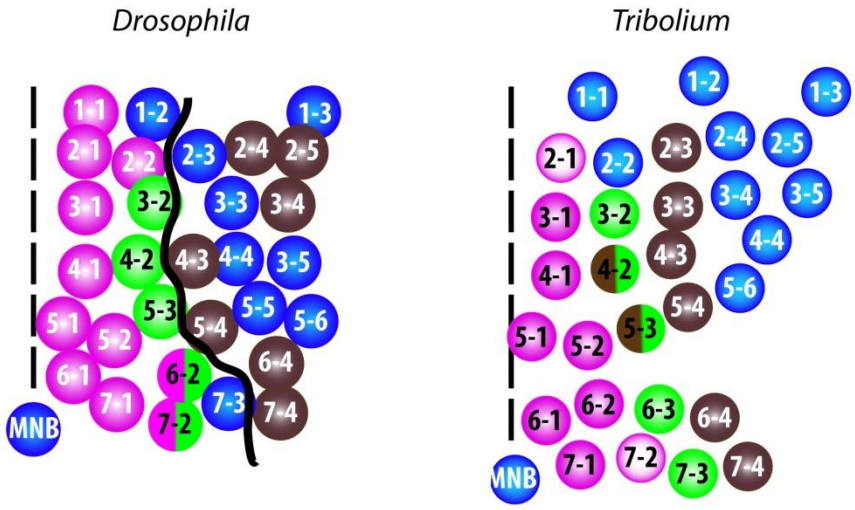


Figure 4-4: Columnar gene expression in *Drosophila* and *Tribolium*

Final neuroblast maps for *Drosophila* (left) and *Tribolium* (right) depicting the expression of three columnar genes *vnd* (pink), *ind* (green) and *msh* (brown) at any given time. The solid black line in the *Drosophila* map represents the border of *msh* expression in the neuroectoderm, demonstrating that not all neuroblasts delaminating out of *msh*$^{+}$ neuroectoderm eventually express *msh*. Furthermore, the apparent co-expression of *ind* and *vnd* in NBs 6-2 and 7-2 is only true for NB 6-2. NB 7-2 has already ceased *ind* expression by the time *vnd* expression commences. The midline is indicated by the vertical dashed line.

The expression of columnar genes in the neuroectoderm of *Tribolium* and *Drosophila* varies more significantly than originally suggested by Wheeler *et al.* (2005). It appears that in *Tribolium Tc-vnd*, *Tc-ind* and *Tc-msh* are expressed only in neuroectodermal cells out of which columnar gene expressing neuroblasts arise. This is in contrast to *Drosophila* where all three columnar genes are initially expressed in continuous domains along the entire neuroectoderm, yet not all neuroblasts arising from these domains necessarily express the respective columnar gene. Furthermore, the three columnar genes analysed exhibit differences in their expression in positionally homologous neuroblasts between *Drosophila* and *Tribolium*. In *Drosophila* two more genes (*Sox-neuro*, *Dichaete*) are involved in the division of the neuroectoderm into three columns (Cremazy *et al.* 2000, Buescher *et al.* 2002, Overton *et al.* 2002, Zhao and Skeath 2002). *Sox-neuro*, has been demonstrated to be necessary for the formation of intermediate and lateral neuroblasts (Buescher *et al.* 2002). Further work would benefit from analysing the expression of the *Sox-neuro* and *Dichaete* orthologues in *Tribolium*. It may be that in *Tribolium* different combinations of spatial identity genes may have evolved in contrast to *Drosophila*.

The data obtained in the present work supports the hypothesis that changes of spatial specification of neural precursor cells along the dorsal-ventral column may result in evolutionary modifications of the nervous system between different arthropod lineages (Doeffinger and Stollewerk 2010).

Functional studies support the hypothesis that changes in columnar gene expression result in evolutionary modifications of arthropod nervous systems

The evolutionary changes of spatial gene expression along the AP and DV axis observed in the present work suggest that segment polarity genes and columnar genes partially changed their regulatory function. To investigate how these changes influence neuroblast formation, neuroblast identity and neural progeny differentiation functional studies for *Tc-vnd*, *Tc- ind* and *Tc-msh* were performed. Wheeler *et al.* (2005) previously demonstrated that in *Tribolium vnd* functions in a similar way as in *Drosophila*. Loss of *Tc-vnd* in *Tribolium* results in the failure of ventral neuroblast formation. Furthermore, *Tc-ind* expression in *Tribolium* is inhibited by *Tc-vnd* in the medial column neuroectoderm, as is also observed in *Drosophila* (Chu *et al.* 1998, McDonald *et al.* 1998, Wheeler *et al.* 2005). The present work has supported the findings of Wheeler *et al.* (2005) and generated further insights regarding the function of *Tc-vnd* in specifying neural identity in *Tribolium*. However, the synthesised double stranded *Tc-vnd* RNA contains a highly conserved homeobox, therefore off-target effect are within the realms of possibility. Nevertheless, the specificity and consistency of the RNAi effects obtained in this work, with previous results in *Tribolium* (Wheeler et al. 2005) and *Drosophila* (Chu *et al.* 1998, McDonald *et al.* 1998, Mellerick and Modica 2002), suggest that off-target effects are not occurring.

The widely used neuron differentiation markers Even-skipped (see Doe *et al.* 1988b, Duffy *et al.* 1991, Chu-LaGraff and Doe 1993, Skeath *et al.* 1995, Mellerick and Modica 2002) and Tailup were used as an indicator of changes in neural identity. Furthermore, the present work revealed that there are significant differences of Eve and Tup expression between wild type *Tribolium* and *Drosophila*.

Number of Tup and Eve expressing neurons in *Tribolium* is larger than in *Drosophila*

The specific expression of Even-skipped in a distinct subset of neurons makes it a suitable marker to analyse changes in neuronal fate. In *Drosophila* embryos it is expressed in around 16 neurons consisting of the aCC/pCC, RP2, U/CQ neurons and the Eve lateral cluster (Fig. 4-4; Doe 1988b, Patel 1989). Duman-Scheel and Patel (1999) claim to have analysed Even-skipped expression in 18 insect species, including *Tribolium*,

but only present data for *Schistocerca*. They, furthermore, demonstrate that the expression pattern of Eve in aCC/pCC, RP2 and U/CQ neurons and the El cluster is conserved between hexapods and crustaceans. The data obtained in the present work demonstrate that the expression of Eve in *Tribolium* is similar to *Schistocerca* and *Drosophila*. However, an additional late forming lateral Eve expressing cluster was detected in the posterior part of each hemineuromere. Due to its position it was termed posterior Eve lateral cluster (PEL). This cluster has not previously been described in *Drosophila*. In *Schistocerca* the existence of an additional posterior lateral Eve expressing cluster was mentioned by Broadus and Doe (1995). Yet, Duman-Scheel and Patel (1999) do not describe the existence of such an additional cluster, either in *Schistocerca* or *Tribolium*. Furthermore, they claim that in some insects, including *Tribolium*, pCC is positioned medially to the aCC neuron and that aCC and pCC can be distinguished from each other by a stronger Eve expression in pCC. In the present work neither the medial position of pCC nor a difference in expression were observed. However, as aCC/pCC neurons migrate from the anterior part of one segment to the posterior part of the anterior lying segment, aCC is not always positioned strictly anterior to pCC. It is likely that in some cases different relative positions occur during the migration process. Therefore, it appears that what Duman-Scheel and Patel (1999) describe as the normal pattern may only be a transient deviation from the normal state. Additionally, these authors analysed the expression of Eve in the collembolan *Folsomia candida*. Collembola (springtails) are one of the three early diverging lineages of hexapods (Trautwein *et al.* 2012). An additional posterior lateral cluster was not described in the collembolan, or any of the crustaceans analysed (Duman-Scheel and Patel. 1999). Therefore one possibility is that the common ancestor of *Schistocerca* and *Tribolium* evolved this additional posterior lateral cluster and that it was lost again in the dipteran lineage. However, as there is no detailed description available for the second lateral cluster in *Schistocerca*, it is impossible to draw any conclusions on the origin and accordingly homology of the PEL cluster in *Tribolium* and *Schistocerca.*

Broadus *et al.* (1995) analysed the expression of *even-skipped* in *Drosophila* in detail, presenting the temporal sequence of when specific GMCs and neurons commence *eve* expression. The first two Eve^{+} cells are the first GMCs generated by NBs 1-1 and 7-1 respectively (GMCs 1-1a and 7-1a). GMC 4-2a (the first progenitor cell of NB 4-2) is only

detected once the latter two GMCs have divided into aCC/pCC and the first U/CQ neurons, respectively. In *Tribolium*, in contrast, the first two Eve^{+} cells are the GMCs, which eventually divide into the aCC/pCC neurons and the RP2 and its sibling neuron (equivalent to GMC 1-1a and 4-2a in *Drosophila*). In *Drosophila* the U/CQ neurons are initially positioned anterior to the aCC/pCC neurons. Eventually aCC/pCC are positioned dorsal/apical to the U/CQ neurons. In *Tribolium* the aCC/pCC neurons have already migrated when the first U/CQ neurons are formed. Therefore they are formed almost ventrally to them. Nevertheless, although early changes in the time of formation occur the final pattern is strikingly similar. Broadus *et al.* (1995), furthermore, detected an additional neuron, the fpCC neuron (friend of pCC) a progenitor cell of NB 7-1 (Bossing *et al.* 1996b, Schmid *et al.* 1999) in the final pattern of Eve expression in *Drosophila*. The fpCC neuron is positioned directly ventrally/basal to the aCC/pCC neurons. In *Tribolium* five neurons basal to the aCC/pCC neurons express *Tc*-Eve. According to their position and arrangement they resemble the U/CQ neurons in *Drosophila*. One of them may be the equivalent of the fpCC neuron. However, it was not possible in the present work to distinguish between U/CQ neurons and a potential fpCC neuron solely by analysing cellular position. The expression of Eve in the fpCC neuron has only been described in *Drosophila* (Broadus *et al.* 1995) and in no other hexapods or crustaceans (Duman-Scheel and Patel 1999). Furthermore, conflicting data on the number of U/CQ neurons in *Drosophila* exist, ranging from four to six cells (Broadus *et al.* 1995, Duman-Scheel and Patel 1999, Schmid *et al.* 1999). In *Tribolium* no more than five neurons were counted. Further ambiguity exists regarding the final number of cells forming the EL cluster in *Drosophila*. Numbers varying from five cells (Schmidt *et al.* 1997), eight to ten cells (Broadus et al.1995) and up to 13 cells (Schmid *et al.* 1999) have been reported. Lineage data in *Drosophila*, however, demonstrate that in contrast to the conserved number of neuroblasts from individual to individual and segment to segment, the number of neurons varies in different segments (Schmid *et al.* 1999). The present work demonstrates that in *Tribolium* the EL cluster consists of around 10 EL cells on average but up to 13 cells were counted in individual hemineuromeres. Thus the number of EL cells appears to be conserved between *Drosophila* and *Tribolium*.

However, the overall number of *Tc*-Eve expressing cells is considerably larger in *Tribolium* (on average 22 cells) than in *Drosophila* (on average 16 cells). This is mainly a result of the additional PEL cluster.

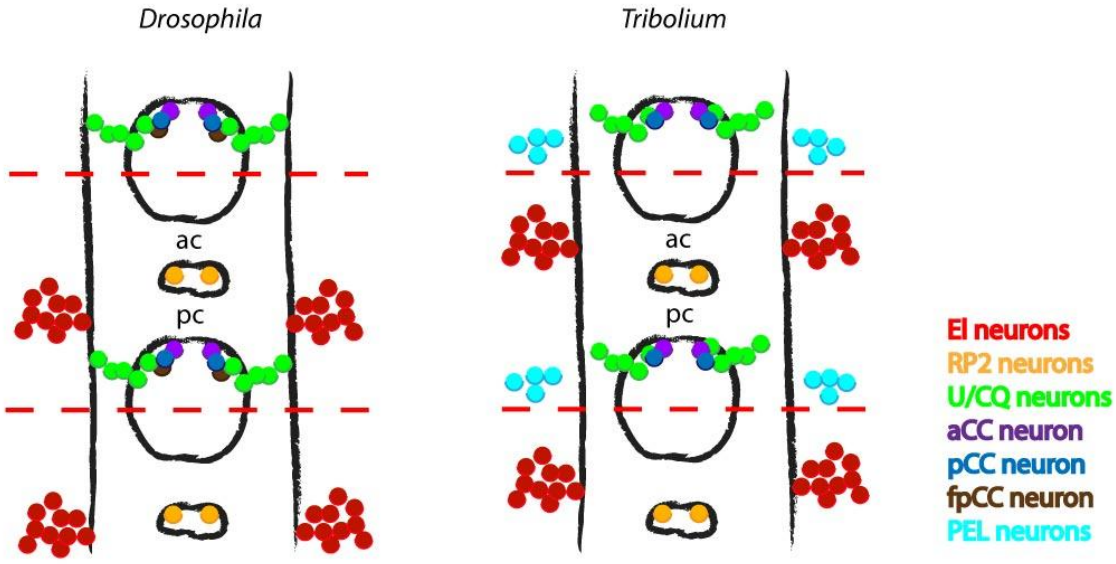


Figure 4-5: Even-skipped expression in *Drosophila* and *Tribolium*

Comparison of neurons that express Even-skipped in *Drosophila* and *Tribolium*. The black lines indicate the axonal scaffold; the segment borders are indicated by the red dashed line; anterior commissure (ac); posterior commissure (pc). Illustration modified after Broadus *et al.* (1995) and Mellerick and Modica (2002).

An additional neural marker used in the present work is Tailup (formerly known as Islet). It is expressed in around 20 to 30 neurons in *Drosophila* (Thor and Thomas 1997). In contrast to the Eve$^+$ neurons only a subset of the Tup$^+$ neurons have been traced back to their origin (Fig. 4-5). NB 3-1 generates the RP1, 3 and 4 motor neurons in *Drosophila* (Landgraf *et al.* 1997). Additionally, Tup expression is detected in multiple interneurons, of which a subset constitute the three dopaminergic and four serotonergic interneurons per segment (Lundell and Hirsch 1994; Thor and Thomas 1997). As previously described, the only four serotonergic neurons per segment are progenitor cells of NB 7-3 (Lundell *et al.* 1996). Of the three dopaminergic cells per segment only the origin of the H-cell, which is generated by midline progenitor cell 3 (MP3), has been proven (Goodman *et al.* 1981, Bossing and Technau 1994). The other two dopaminergic interneurons are likely to be progenitor cells of NBs 5-1 and 5-6 (Tio *et al.* 2011). In *Tribolium* around 50 Tup$^+$ neurons

were detected per hemineuromere, the majority of which were positioned in the anterior part of the hemineuromere. Furthermore, an unpaired cell along the midline was detected, resembling the H-Cell. Additionally, neurons in the position of the RP neurons were detected. It appears that part of the Tup expression in neurons is conserved between *Tribolium* and *Drosophila*. However, there are approximately twice as many Tup^+ neurons in *Tribolium* than in *Drosophila.*

One explanation for this may be that the increase in Tup^+ and Eve^+ neuron numbers in *Tribolium* is an implication of a higher overall number of inter- and motor neurons compared to *Drosophila*. In contrast to *Drosophila, Tribolium* larvae exhibit thoracic appendages. It can be assumed that moving and coordinating the additional muscles of these appendages requires a greater complexity of the thoracic nervous system in comparison to the *Drosophila* larva which is restricted to body movement. This increase in complexity of movement may be reflected in a larger number of neurons. However, this hypothesis remains to be tested, as an increase of Tup^+ and Eve^+ neurons may not necessarily indicate an increase in the overall number of neurons. Analysis of additional genes known to be expressed in *Drosophila* neurons is required to assess the validity of this hypothesis.

Tc-vnd is required for the formation of several ventral neuroblasts and regulates neuroblast identity and lineage differentiation

As mentioned above, loss of *Tc-vnd* results in the failure of ventral neuroblast formation (Wheeler *et al.* 2005). Previously it was not possible to identify neuroblasts that failed to form. In the present work it was possible to demonstrate the loss of a number of ventral neuroblasts in *Tc-vnd*^{RNAi} embryos. Furthermore, analysing the expression pattern of the neuronal markers Eve and Tup demonstrates that *Tc-vnd* regulates neuroblast identity which in turn determines and regulates the neuroblast lineages. As *Tc-vnd* is also expressed in neurons it may be that it directly regulates Eve and Tup expression in neurons. The results suggest that *Tc-vnd* function has partially diverged between *Drosophila* and *Tribolium.*

Alterations in the expression pattern of *Tc-ind* and *Tc-cas* suggest that ventral neuroblasts of row three, four, five and six are missing in *Tc-vnd*^{RNAi} embryos. *Tc-ind* is normally expressed in a single column of intermediate neuroblasts separated from the midline by

one to two columns of ventral *Tc-vnd* expressing neuroblasts (Fig. 4-3). In *Tc-vnd*^{RNAi} embryos the number of *Tc-ind* neuroblasts is unchanged but the *Tc-ind*$^{+}$ neuroblasts are positioned directly along the midline with no neuroblasts medially to them. *Tc-cas* is initially expressed in ventral neuroblasts. This expression is lost in *Tc-vnd* embryos which, furthermore, implicates that several ventral neuroblasts are not formed. However, the lack of *Tc-cas* expression is not necessarily an indication for the loss of neuroblasts. It could imply a change of ventral neuroblast identity to intermediate neuroblast identity. In combination with the *Tc-ind* results, however, it appears more likely that the absence of *Tc-vnd* expression is caused by a lack of ventral neuroblasts.

The combination of neuroblast marker genes (*Tc-ind* and *Tc-cas*) in combination with the neuron markers *Tc*-Eve and *Tc*-Tup permit to conclude which specific ventral neuroblasts may not be formed. In *Tc-vnd* embryos no expression of *Tc*-Eve in U/CQ or *Tc*-Tup in RP 1, 3, 4 neurons was detected. Assuming that in *Tribolium* and *Drosophila* U/CQ and the RP1, 3, 4 neurons are formed by homologous neuroblasts, the data suggests that NB 7-1 (precursor of U/CQ neurons) and 3-1 (precursor of RP1, 3 and 4 neurons) are either not formed, undergo an identity change or exhibit differentiation defects. If the latter was the case, the neurons generated by these neuroblasts may also show differentiation defects or not form at all. In *Drosophila,* ventral neuroblasts (1-1, 2-2, 3-1, 4-1, 5-1, 5-2 and 7-1) are mostly missing in *vnd* mutants except for NBs 2-1 and 6-1 (Chu *et al.* 1998). The expression of *vnd* in the positionally equivalent neuroblasts 3-1 and 7-1 is conserved between *Tribolium* and *Drosophila* (Fig. 4-3). The conserved expression of *Tc-vnd* suggests a similar function in these neuroblasts. Thus it appears very likely that the failure of detecting Eve and Tup expression in U/CQ and RP neurons is caused by the failure of formation of NBs 7-1 and 3-1.

In addition to loss of *Tc*-Eve expression in the U/CQ neurons in *Tc-vnd*^{RNAi} embryos, further phenotypes of *Tc*-Eve expression concerning aCC/pCC neurons were observed. In 90% of cases *Tc*-Eve was detected in aCC/pCC neurons in *Tc-vnd*^{RNAi} embryos. This is in stark contrast to *Drosophila* where Eve^{+} aCC/pCC neurons are never detected in *vnd* mutants (Chu *et al.* 1998, McDonald *et al.* 1998, Mellerick and Modica 2001). The lack of aCC/pCC neurons in *Drosophila* is not necessarily caused by a failure of NB 1-1 formation but can be the result of an identity change of NB 1-1 (Chu *et al.* 1998,

McDonald *et al.* 1998). Furthermore, in 1/3rd of cases only one progenitor of NB 1-1 was detected expressing *Tc*-Eve in *Tc- vnd*^{RNAi} embryos. In over 60% of hemineuromeres both aCC and pCC were formed. Yet, in 15% of these cases aCC/pCC exhibited altered migration patterns. Migration defects were observed in almost half of the cases where only on NB1-1 progeny was formed. Thus in 90% of hemineuromeres at least one progeny of NB 1-1 was detected expressing *Tc*-Eve. Therefore it can be assumed that the missing Tc-Eve expression is not the result of a failure of formation of NB 1-1 but rather results from differentiation defects caused by the lack of *Tc-vnd* transcripts.

A question remaining is which progenitor cell constitutes the single cell generated by NB 1-1 in some *Tc-vnd*^{RNAi} embryos. It could be either the GMC, which fails to differentiate into aCC/pCC neuron or the aCC or pCC neuron. In almost fifty percent of cases when only one cell is formed this cell does not migrate. Assuming that only the aCC/pCC neurons have the ability to migrate would suggests that the single cell is the GMCs. It is, however, questionable if the division pattern of the GMC is influenced by *Tc-vnd* as none of the columnar genes in *Drosophila* have been reported to influence GMC division.

The differences described above between *Tribolium* and *Drosophila* appear to be the result of a change in *vnd* expression in NB 1-1. As discussed before *Tc-vnd* expression is not maintained in NB 1-1 after its delamination. The functional results show that this short-term activity of *Tc-vnd* is not required for the formation of NB 1-1 as in *Drosophila* rather it is sufficient for conferring neuroblast identity which in turn affects the differentiation of the neuronal progeny. However, it can not be ruled out that *Tc-vnd* is expressed later in the aCC and pCC neurons and directly regulates neuronal differentiation genes.

Alternatively the RNAi phenotype could result from a combination of both scenarios. In *Drosophila* it was demonstrated that not only NB 1-1 but also the pCC interneuron expresses *vnd* whereas the sibling neuron aCC does not express *vnd*. The expression in neurons appears to be independent of the early expression in the neuroectoderm and neuroblasts (McDonald *et al.* 1998). The presented data show that *Tc-vnd* is also expressed in neurons in *Tribolium*, although the expression could not be related to identified neurons. The frequent loss of one of the aCC/pCC siblings in *Tc-vnd*^{RNAi} embryos would suggest that *Tc-vnd* is expressed in pCC and the neuron fails to differentiate properly in the absence of *Tc-vnd* function. This data supports a late function

of *Tc-vnd* in the NB1-1 lineage. Furthermore, if we assume that *Tc-vnd* is not expressed in aCC similar to *Drosophila*, the migration defect of aCC (and possibly pCC) in RNAi embryos must result from a lack of the early transient expression of *Tc-vnd* in NB1-1.

In addition to the phenotypes described above a second RP2 neuron is detected in some *vnd* mutants in *Drosophila*. The RP2 neuron is normally formed by the intermediate NB 4-2. Lacking *vnd* transcript a ventral neuroblast of row three, four or five changes its identity to NB 4-2 and generates the additional RP2 neuron (Chu *et al.* 1998). In *Tribolium* no second RP2 neuron was detected. The easiest explanation is that no ventral neuroblasts are formed and therefore they can not change their ventral neuroblast identity towards intermediate neuroblasts identity. Another possibility would be that further genes are involved in conferring ventral neuroblast identity and are able to rescue the identity in case a ventral neuroblast is formed in a *Tc-vnd* negative environment. This gene could be *Dichaete.* In *Drosophila Dichaete* confers medial and intermediate neuroblast identity. In *Dichaete* mutants NB 4-1 fails to form in 30% of cases (Zhao and Wheeler 2002). Neuroblast 4-1 is one of the likely neuroblasts to take on NB 4-2 fate in *vnd* mutants, generating the second RP2 neuron (Chu *et al.* 1998). Assuming that NB 4-1 in *Tribolium* is also dependent on *Dichaete* expression for its formation and specification could mean that in contrast to *Drosophila*, *Dichaete* in *Tribolium* rescues NB 4-1 identity and does not allow it to take on NB 4-2 identity, thereby no second RP2 neuron is formed. However, these are mere speculations as nothing is known about the expression of *Dichaete* in *Tribolium*.

Functional studies of *Tc-ind* and *Tc-msh*

It was previously demonstrated that *Tc-ind* is essential for the formation of intermediate neuroblasts in *Tribolium* (Wheeler *et al.* 2005). In the current work analysis of *Tc-ind* was undertaken in order to investigate its role in specifying neural identity in neuroblast and thus in influencing the differentiation of neuronal progenitor cells. In *Drosophila* only 10% of intermediate neuroblasts form in *ind* mutants. Additionally, the RP2 neuron generated by NB 4-2 is missing in *ind* mutants. This phenotype is either caused by a failure of formation of NB 4-2 or by division and specification defects of NB 4-2 (Weiss *et al.* 1998). Although, *Tc-ind* expression was strongly reduced in *Tc-ind*^{RNAi} embryos no impact on *Tc*-Eve expression was observed (see Chapter 3-3). According to Wheeler *et al.* (2005) 80%

of intermediate neuroblasts are not formed in *Tc-ind*^{RNAi} embryos, therefore a loss of NB 4-2 and its daughter cell, the RP2 neuron, was expected. The results reported here are based on a relatively small number of RNAi embryos (n=4) and therefore have to be confirmed by additional function studies.

In contrast to both *vnd* and *ind*, *msh* is not required for the formation of neuroblasts but is essential for proper development of lateral neuroblasts and their progenitor cells (Isshiki *et al.* 1997). Differentiation defects in neurons and glial cell are detected in *msh* mutant *Drosophila* embryos. Wheeler *et al.* (2005) suggested that *Tc-msh* functions in a similar way in *Tribolium*. However, the data provided to support this hypothesis was incomplete. Firstly, no data demonstrating the down-regulation of *Tc-msh* in *Tc-msh*^{RNAi} embryos was presented. Secondly, no studies were performed concerning the effects of *Tc-msh* loss on neuronal differentiation of lateral neuroblast progenies. Therefore the aim of the work presented here was to analyse embryos lacking *Tc-msh* expression, to initially confirm its non-essential role in neuroblast formation, and subsequently to analyse its function in neuronal precursor differentiation. However, none of the four dsRNAs generated, varying in length and target sequence, could reduce *Tc-msh* expression, either through pupal or embryonic RNAi. In all cases *Tc-msh* was expressed as in wild type embryos.

In the chelicerate *Cupiennius salei*, *msh* is not involved in the formation of NPGs, but appears to function in neuronal precursor differentiation (Doeffinger and Stollewerk 2010), similar to its role in *Drosophila*. However, in *Cupiennius tailup* expression was absent in *msh*^{RNAi} embryos, demonstrating a role of *msh* in regulating *tailup* expression in neurons differentiating out of lateral neural precursor cells. In *Drosophila tailup* expressing neurons are generated mainly by medial and intermediate column neuroblasts and therefore are not under the influence of *msh* (Fig. 4-6; Thor and Thomas 1997). In *Tribolium* the number of Tup^{+} cells is at least 1/3 larger than in *Drosophila*, as mentioned above. Furthermore, Tup^{+} cells are positioned in the medial, intermediate and lateral domain of a hemineuromere (Fig. 4-6). Although it was not possible to correlate Tup neurons to their precursor neuroblasts, other than for the H-cell and RP neurons, the work presented here suggests that some of the Tup^{+} cells are formed by ventral neuroblasts as discussed before. Furthermore, it appears likely that some Tup^{+} cells are generated by lateral and intermediate neuroblasts. Therefore it may be the case, that in *Tribolium* some *Tup*$^{+}$

neurons are formed by *Tc-msh*$^{+}$ neuroblasts and their differentiation may be regulated by *Tc-msh*. The data from *Cupiennius* and *Drosophila* suggest that the general function of *msh* during differentiation processes is conserved within arthropods. However, different downstream genes are regulated in *Drosophila* and *Cupiennius*. Therefore it is very likely that, as suggested by Wheeler *et al.* (2005), *Tc-msh* functions during differentiation processes in some lateral neuroblasts and their progenitor cells but is not involved in their formation, similar to its role in *Drosophila* and *Cupiennius*. *Tribolium* appears to represent a state between *Drosophila* and *Cupiennius* with Tup^{+} cells being generated by ventral, intermediate and lateral neuroblasts.

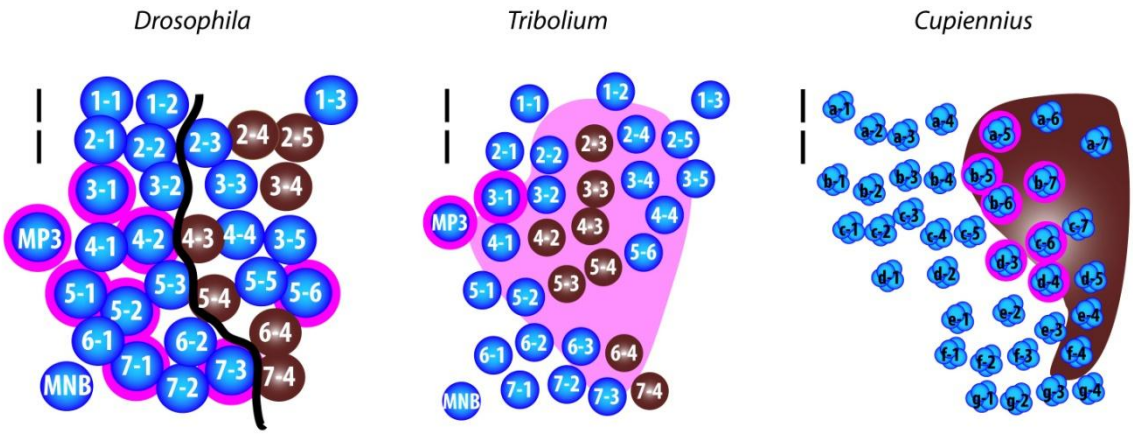


Figure 4-6: Comparison of *msh* and Tup expression in *Drosophila*, *Tribolium* and *Cupiennius*

msh expression is depicted in brown; Tup expression is depicted in pink. The area where Tup+ cells can be found in *Tribolium* is shown in light pink. In *Drosophila* Tup expressing neurons are generated by the neuroblasts shown with a pink outer circle. In *Tribolium* Tup cells could not be correlated to their precursor cells, except for the MP3 and NB 3-1 (shown with pink outer circle). Nevertheless, the position of Tup^{+} cells in the hemineuromere permits speculation regarding which neuroblasts may be the precursor cells. In *Cupiennius* all Tup^{+} neurons differentiate out of lateral neural precursor groups expressing *msh*. The midline is indicated by the dashed line.

Although complete functional studies for *Tc-ind* and *Tc-msh* were not possible in the present work, differences in their expression pattern between *Drosophila* and *Tribolium* were observed, suggesting that their function may have slightly changed over evolution. Furthermore, correlating the expression of the neural marker gene *tailup* to *msh* expression shows that the function of columnar genes on neuronal subtype specific genes has altered over evolution. In addition, the expression and function of the columnar gene *Tc-vnd* demonstrates how the function of a gene has evolved differently between two insect species. In contrast to *Drosophila Tc-vnd* in *Tribolium* appears to be unnecessary for the formation of NB 1-1. The reduction in *Tc-vnd* expression and the resulting difference in neuron formation is an example where a subtle change in gene expression may cause a different function of an orthologous gene in different insect species.

Summary and Outlook

In the current work early neurogenesis in the coleopteran *Tribolium castaneum* was investigated and the results were compared primarily to the dipteran *Drosophila melanogaster*. Neuronal mechanisms which are common to both insects, and mechanisms which have diverged, were thereby revealed.

Firstly it was demonstrated that the general arrangement of neuroblasts is largely conserved between *Tribolium* and those insect species previously investigated. Minor changes in the number of neuroblasts and the timing of their formation were observed. These small changes, however, can only account for limited differences between species. Significant differences were revealed in the expression of segment polarity genes and columnar genes, which confer neuroblast identity along the AP and DV axis, respectively. Expression of the segment polarity genes in the neuroectoderm was mostly conserved between *Tribolium* and *Drosophila*. However, significant differences in the expression of these genes in positionally homologous neuroblasts of the two species were observed. Furthermore, expression of the columnar genes was shown to diverge both in the neuroectoderm and in neuroblasts.

To analyse the possible effect of these changes on neuroblast identity, the function of the columnar gene *Tc-vnd* was studied in greater detail. The present work demonstrated that small changes in *Tc-vnd* gene expression pattern can result in differing functions in neuronal differentiation. Additionally, analysis of the neuronal marker genes *even-skipped* and *tailup* revealed differences in the number of neurons in which expression was detected between *Tribolium* and *Drosophila*. These changes of expression pattern in neuroblasts and neurons may allow for adaptation of the nervous system to species specific morphology and behaviour.

By producing a neuroblast map for *Tribolium* it was possible to demonstrate differences of gene expression in positionally homologous neuroblasts. Lineage analyses will reveal whether these neuroblasts are genuine homologues that altered their gene expression pattern or if in some cases homologues neuroblasts might have changed their position within the neuroblast array between *Tribolium* and *Drosophila*.

Positionally homologous neuroblasts that exhibit differing gene expression pattern between *Tribolium* and *Drosophila* will have to be analysed in detail to understand what these alterations result in. In the current work mainly the function of the columnar genes, with a special focus on *Tc-vnd*, was analysed in detail. For all other neural identity genes only their expression pattern was described and compared to *Drosophila*. Therefore, functional studies of the neural identity genes that exhibit differences in their expression pattern between *Drosophila* will have to be performed in the future. A gene of special interest is *wg* which is expressed in all row five neuroblasts in *Drosophila*. Row five neuroblasts, however, are not dependent on its expression. The secreted Wg protein confers a unique identity to row three, four and six neuroblasts (Chu-LaGraff and Doe 1993, Bhat 1996, Bhat and Schedl 1997). In the current work it was demonstrated that *Tc-wg* in *Tribolium* is only expressed in three of five row five neuroblasts. Therefore it will be very interesting to investigate the role of Wg protein in influencing the identities of neuroblasts in different rows, as seen in *Drosophila*, and what further implication on diversification of the insect nervous system these differences may have. Eventually functional studies of different neural identity genes will demonstrate what effect differences in the expression pattern of neural identity genes may have on neural identity and differentiation of neural precursor cells.

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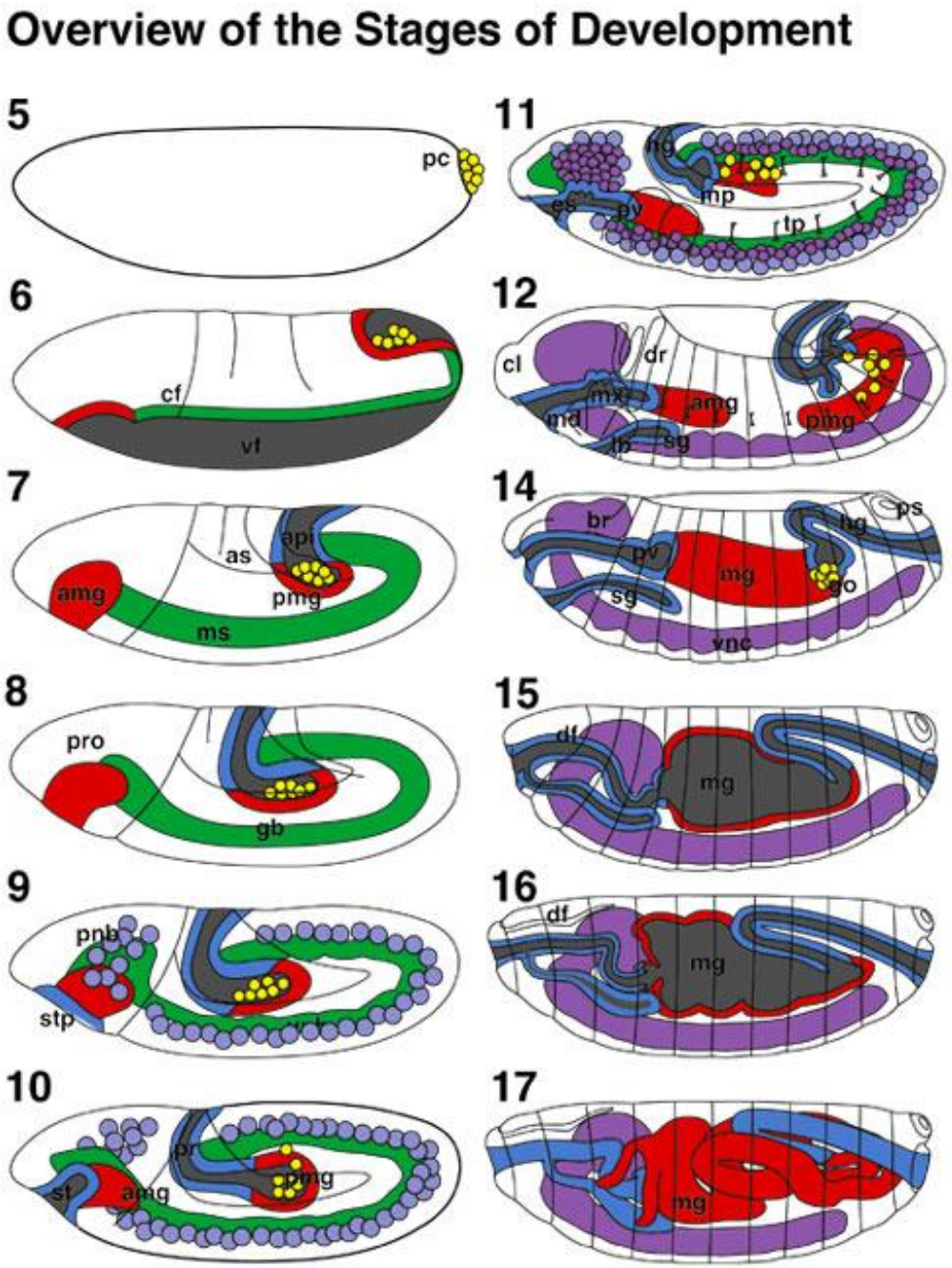
Appendix

Drosophila staging



“All embryos are in lateral view (anterior to the left). Endoderm, midgut; mesoderm; central nervous system; foregut, hindgut and pole cells in yellow.(*amg*) (Anterior midgut rudiment; (*br*) brain; (*cf*) cephalic furrow; (*cl*) clypeolabrum; (*df*) dorsal fold; (*dr*) dorsal ridge; (*es*) esophagus; (*gb*) germ band; (*go*) gonads; (*hg*) hindgut; (*lb*) labial bud; (*md)* mandibular bud; (*mg*) midgut; (*mg*) Malpighian tubules; (mx) maxillary bud; (*pc*) pole cells; (*pmg*) posterior midgut rudiment; (*pnb*) procephalic neuroblasts; (*pro*) procephalon; (*ps*) posterior spiracle; (*po*) proventriculus; (*sg*) salivary gland; (*stp*) stomodeal plate; (*st*) stomodeum; (*tp*) tracheal pits; (*vf*) ventral furrow; (*vnb*) ventral neuroblasts; (*vnc*) ventral nerve.” (Hartenstein 1993)

Stage 1-4: cleavage; stage 5: blastoderm; stage 6-7: gastrulation; stage 8-11: germ band elongation; stage 12-13: germ band retraction; stage 14-15: head involution and dorsal closure; stage 16-17: differentiation

Staging of *Drosophila* taken from *Atlas of Drosophila Development* by Volker Hartenstein 1993 published by Cold Spring Harbor Laboratory Press, 1993; page 52**;** http://www.sdbonline.org/fly/atlas/00atlas.htm

RNA interference

msh RNAi in pupae

***msh* fragment used for RNA probe (concentration of injected RNA in ng/ µl; see M&M page 42)**	**Pupae injected**	**Pupae hatched**
2.6 ng/µl	43	10
2.4 ng/µl	41	36
2.5 ng/µl	40	27
2.1 ng/µl	92	20
2.7 ng/µl	35	30
2.5 ng/µl	43	35
1.6 ng/µl	121	70
Total	**415**	**228**

Control A (buffer injection)	86	40
Control B ds *ubx* RNA 2.3 ng/µl	125	13

msh embryonic RNAi

***msh* fragment**	**Embryos injected**	**Embryos survived**
msh fragment used for RNA probe (see M&M page 42)	298	70
	279	171
msh short 1 (195bp) (see M&M page 43)	283	140
	303	79
	278	56
msh short 2 (170bp) (see M&M page 43)	120	38
msh long (870bp) (see M&M page 43)	98	20

ind embryonic RNAi

***ind* fragment**	**Embryos injected**	**Embryos survived**
ind fragment used for RNA probe (see M&M page 42)	303	79

vnd embryonic RNAi

Embryos injected	**Embryos survived**	**Age of embryos when fixed in hours**
286	33	19
320	123	21½-23
248	128	30
304	30	28½-30; 42½-44
268	85	31½-30
100	30	34½-36

vnd expression in *vnd*RNAi embryos

total number of embryos	11

no *vnd* expression	4
faint *vnd* expression	2
vnd expression in abdominal segments	4
expression as in wild type	1

Solutions

Unless otherwise noted, all solutions were made with deionised water and autoclaved.

Hybridisation buffer

Deionized formamide 50% (v/v) (5g mixed bead resin in 50 ml formamide. Mix for 1 h.)

2x SSC (10 ml 20xSSC)

0.1% Tween-20 (100 µl)

50 µg/ml Yeast tRNA (250 µl 20 mg/ml)

50 µg/ml heparin (250 µl 20 mg/ml)

DDW to 100 ml

Injection buffer *Tribolium*

5 M NaCl

10 mM Na_2HPO_4

10 mM KH_2PO_4

100 mM KCl

Fill to 50 ml H_2O

Store at -20˚C

10x PBS (Phosphate buffered saline) stock solution

1.3 M NaCl
2.7 mM KCL
1.8 mM KH_2PO_4
10 mM Na_2HPO_4
pH 7.4
Fill to 1000 ml with H_2O

PBT (PBS + 0.02% Tween)

100 ml 10x PBS
1 ml 10% Tween 20
Fill to 1000 ml with H_2O

PBTrit (PBS + 0.1% Triton)

100 ml 10 x PBS
1 ml 10% Triton-X 100
Fill to 1000 ml with H_2O

PEMS
100 M PIPES
1 mM EDTA
2 mM $MgSO_4$
pH 6.9

20 x SSC

3 M NaCl
0.3 M sodium citrate
pH 7 (with 14N HCL)

Alkalic phosphatase detection buffer (prepare fresh)

100 mM NaCl

50 mM $MgCl_2$

100 mM Tris-HCl

0.01% Tween-20

pH 9.5

Fast Red staining buffer (pH 8.2 Tris-HCl buffer)

100 mM Tris-HCl pH 8.2

0.1% Tween-20

TAE-stock solution (Tris-Acetat-EDTA), pH 8.3

242 g Tris in 500 ml H_2O

100ml 0.5 M Na_2EDTA, pH 8

57.1 ml glacial acetic acid

add water to 1 l

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